Temperature drives P granule formation in *Caenorhabditis elegans*

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Declaration of Authorship

Declaration according to §5.5 of the doctorate regulations

I, Andrés Felipe Diaz Delgadillo, declare that I have produced this thesis titled, '**P granule formation is thermodynamically driven by Temperature in *Caenorhabditis elegans***' without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from 01.11.2011 to 01.09.2015 under the supervision of Prof.Dr. Anthony Hyman at the Max Planck Institute of Molecular Cell Biology and Genetics.

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Andrés Felipe Diaz Delgadillo, October 2015

Dedicated to my three beloved girls, Janina, Enie and Élena.

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1. Abstract

Ectotherms are living creatures whose body temperature varies with the environment in which they live. Their physiology and metabolism have to rapidly respond to environmental changes in order to stay viable at across their tolerable thermal range (Lithgow *et al.* 1994). In nematodes such as *Caenorhabditis elegans*, temperature is an important factor that defines the fertility of the worm. A feature that delimits an ectotherm's thermal range is the maximum temperature at which its germ line can produce gametes. How germ cells withstand high environmental stressors such as limiting temperatures is not well understood, especially when considering the thermodynamical principles that dominate the biochemical processes of the cytoplasm (Hyman and Brangwynne 2011).

Previous studies in *C. elegans* have shown that the thermodynamic effects of temperature on the cell cycle rate in nematodes follows an Arrhenius relationship and defines the thermal range where worms can be fertile. At the limits of this relationship a breakdown of the Arrhenius trend is observed (Begasse *et al.* 2015a). It was hypothesized that some type of discontinuous phase transition occurred in the embryonic cells of *C. elegans* (Begasse et al 2015). However, it remains unknown if there is the physiological link between a drop off in fertility and the embryonic breakdown of the Arrhenius trend.

This work finds the link between a temperature driven phase separation of P granules and fertility. P granules are important for germ line development and the fertility of *C. elegans* (Kawasaki *et al.* 1998b). Here it is shown that P granules mix with the cytoplasm upon a temperature quench of 27°C to T=18°C and de-mix from the cytoplasm forming droplets upon a temperature downshift of temperature from 18°C to 27°C. P granules also show a reversible behavior mixing and de-mixing with changes in temperature *in vivo,* having a strong dependence of these liquid-like

compartments with entropy. These results were further confirmed using a minimally reconstituted, *in vitro* P granule system and showed that PGL-3, a constitutive component of P granules, can phase separate and form liquid compartments in a similar way as happens *in vivo*.

Additionally, here it is shown that P granule phase separation does not require the chemical activity of other cytoplasmic factors to drive the phase separation of compartments *in vivo* and *in vitro*, instead their formation is strongly driven to mix and de-mix with changes in temperature. Furthermore, a binary phase diagram was constructed in order to compare the response of P granules *in vivo* and *in vitro,* showing that P granules form and function as a temperature driven liquid phase separation. Altogether, this indicates that P granules *in vivo* and PGL-3 liquid-like compartments *in vitro*, share the same temperature of mixing and de-mixing which coincides with the fertile temperature range over which *Caenorhabditis elegans* can reproduce. This suggests that P granule phase separation could define the thermal range of the worm.

2. Introduction

All living creatures require a temperature range to optimally grow and multiply at sustainable levels; this is true for single cells and also for higher levels of organization such as multicellular individuals, populations, communities and ecosystems (Schabhüttl *et al.* 2013). Oscillations in temperature affect the way these assemblies organize and respond to new temperatures. Specially, regarding physiological responses involved in the control of embryonic development and the preservation of appropriate functions of cellular factors responsible for producing gametes, such as the germ lineage (Strome and Updike 2015).

The germ lineage is at the center for ectotherm temperature adaptation. Without a proper differentiated germ line ectotherms can not reproduce. Therefore it is important to study the mechanisms by which the germ lineage develops at extreme physiological limits in order to understand how cells molecularly adapt to extreme temperatures.

Differences in temperature, pressure, salinity and pH are the most primitive factors that early life had to tackle in order to adapt to variable conditions. Some remarkable examples are found in the Antarctica where the pshychrophilic nematode *Eudorylaimus sp* thrives at temperatures close to -80°C throughout the whole year (Adhikari *et al.* 2009). In contrast, its polychaetid counterpart *Alvinella pompejana* lives in the depths of oceanic thermal vents where temperature gradients from 22 to 80°C dominating the landscape (Cary *et al.* 1998;Vanreusel *et al.* 2010).

It remains unclear if there are any differences between the germ line of those species that allow them to live and reproduce under such extreme environments. This comparison brings us to a more obvious and striking question: what are the underlying thermodynamic principles that dominate and organize the cytoplasm under such extreme environments specially in the germ line?

Temperature is a crucial aspect of thermodynamics, and its biological role has been kept out of the main stream scientific investigation due its broad physiological effects and difficulty to phenotypically describe its effects *in vivo*. Nevertheless, temperature is important because it influences, in a global manner, many biochemical reactions and physico chemical interactions in the cytosol, placing it at the center of the thermodynamic principles that govern physiology and biochemistry. Examples of this are the effects of temperature in cellular respiration (JOHNSTON *et al.* 1994), cell cycle rates of nematode embryos (Begasse *et al.* 2015a), sex determination in medusae (Carré and Carré 2015) and the effects of membrane bound temperature-dependent gated ion channels (Chowdhury *et al.* 2015).

The influence of temperature on how the cytoplasm biochemistry affected, makes it a tough factor to study because of the increasing amount of chemical reactions and transitions of matter that occur in the cytoplasm. For instance changes in membrane fluidity such as liquid-liquid transitions, gel or glass like as in disease related aggregation processes (Patel *et al.* 2015) or just as simple as the formation of a spindle (Reber *et al.* 2013).

Despite of the biological complexity of the cytoplasm, all metazoans live and organize their cytoplasm by forming structures using ATP as a source of energy to compartmentalize biochemical reactions, this occur including spindles, membranes, P bodies, P granules and other cytosolic compartments. This is achieved independently of the environment where they live, and must involve tuning the thermodynamics of the cell through the regulation of mechanisms such as osmotic pressure, surface tension, and viscosity in order to survive (Stewart *et al.* 2011;Peukes and Betz 2014;Sackmann 2014).

2.1. CYTOPLASMIC ORGANIZATION

One possibility to understand the thermodynamic laws and principles that organize the cytoplasm of the germ line is to study the physics of multi-

component mixtures in soft matter physics. The cytoplasm itself is a complex mixture with different components at very high protein densities. In contrast to non-living industrial polymers, cytoplasmic components, such as DNA and proteins as “living polymers”, exert direct control on the formation of higher macroscopic structures in a coordinated way compartmentalizing cytoplasmic material into organelles such as centrosomes (Zwicker *et al.* 2015) and spindles (Reber and Hyman 2011;Reber *et al.* 2013) with precise spatio-temporal control with the cell cycle.

In recent years a blossoming in the study of organelle formation has led the scientific community to explore the basic mechanisms by which physical forces give shape to the cytoplasm. The question on how organelles form has been considered for more than 100 years (Boveri 1887). Of special interest is the study and formation of spindles and centrosomes, their dynamics throughout the cell cycle (Grill and Hyman 2005;Howard 2006), their rheological properties as the sum of their constituents, their growth rates and limiting components (Reber *et al.* 2013), and how their inter-molecular interactions trigger basic organizational patterns between their components (Woodruff *et al.* 2014;Woodruff *et al.* 2015).

More recently, features such as nucleation control, condensation and growth rates have been described as features proper of “active liquids”, considered to be as those cytoplasmic organelles that consume ATP while resembling a dynamic liquid-like properties *in vivo* such as centrosomes and spindles (Prodon *et al.* 2010;Decker *et al.* 2011;Goehring and Hyman 2012). However, the formation of liquid P granules condensing in a concentration dependent manner (Brangwynne *et al.* 2009a) opens up new possibilities to explore the properties of soft matter physics in biology. These data increase the inventory of thermodynamic mechanisms that control and drive the formation of cytoplasmic compartments by means of a cytoplasmic liquid phase separation.

Living matter organizes its own structure in liquid-like environments such as the cytoplasm. However despite its liquid nature, different organelles follow different organizing rules, depending on the type of intermolecular interactions involved on their assembly. An example of this are the Lewy bodies, that are solid-like

aggregates within dopaminergic neurons linked to Parkinsons's disease (Shtilerman *et al.* 2002) or other pathological aggregates that change their liquid behavior to solid like state aggregation in Amyotropic Lateral Sclerosis (ALS) (Patel *et al.* 2015). The nucleolus itself is liquid-like organelle that regulates its size via mechanisms involving phase separation (Zhu *et al.* 2015). This shows that the transition between different cytoplasmic states can have dramatic consequences for the activity and development of cytosolic organelles involved in certain metabolic tasks.

Most of the efforts in this field had assumed that cells are isothermic systems, where temperature is fixed but the concentration of the components and the volume of the system change. Under this paradigm, energy is provided by ATP and the rate of organelle growth is measured as a function of time or cell cycle length. This experimental view is in part the product of early fundamental questions that allowed the field of cell biology to understand how a cell divides under standard conditions. Today, our inventory of cell parts and their mechanisms have taken us to answer new questions, for example to address how these cellular systems behave under different environmental conditions. Cells are indeed not isothermic systems, quite the opposite, they rely on the change in temperature to trigger physiological reactions of survival and adaptation. In order to understand the thermodynamics of cells in extreme environments would be interesting to study the mechanisms that upkeep cell division at extreme temperatures and compare how different they are from their mesothermic responses to dissect general thermodynamic principles that govern the organization of the cytoplasm. For this, a new use and interpretation of temperature in thermodynamical terms, has to be related to the physiology of living organisms at the cellular level to have a clearer view of how thermodynamical principles operate to build up cells and allow them to adapt and survive at different ecological contexts.

2.2. CYTOPLASMIC PHASE SEPARATIONS

The transition between different cytoplasmic states (gas-like, liquid-like, gel-like, glass-like and solid aggregate) during the lifetime of a cell is key to understand the principles between altered and native functional cytoplasmic states. A way to understand this problem is by bridging the concept of liquid phase separations in cell biology and understand how these transitions affect the biology of the cell and in particular those mechanisms responsible for adaptational responses to stress in the germ line.

In principle, a phase separation occurs when in a homogeneous solution (cytoplasm) of N components; one or more components de-mix from the total volume of the system as a compartment.

The formation of this phase separated region is mainly driven by diffusion of each molecule and their net interaction. In the case of liquid liquid phase separations the interactions between molecules are classified as weak interactions. The energy of mixing for two components reach equilibrium depending on the chemical potential of each of the components in the mixture (designated as ΔFmix, equation I1)).

Free Energy for a Binary Liquid Mixture

$$\Delta F_{mix} = -kbT\,[\,\mu A^{*}\ln(\mu A) + \mu B^{*}\ln(\mu B)\,]$$

Equation I

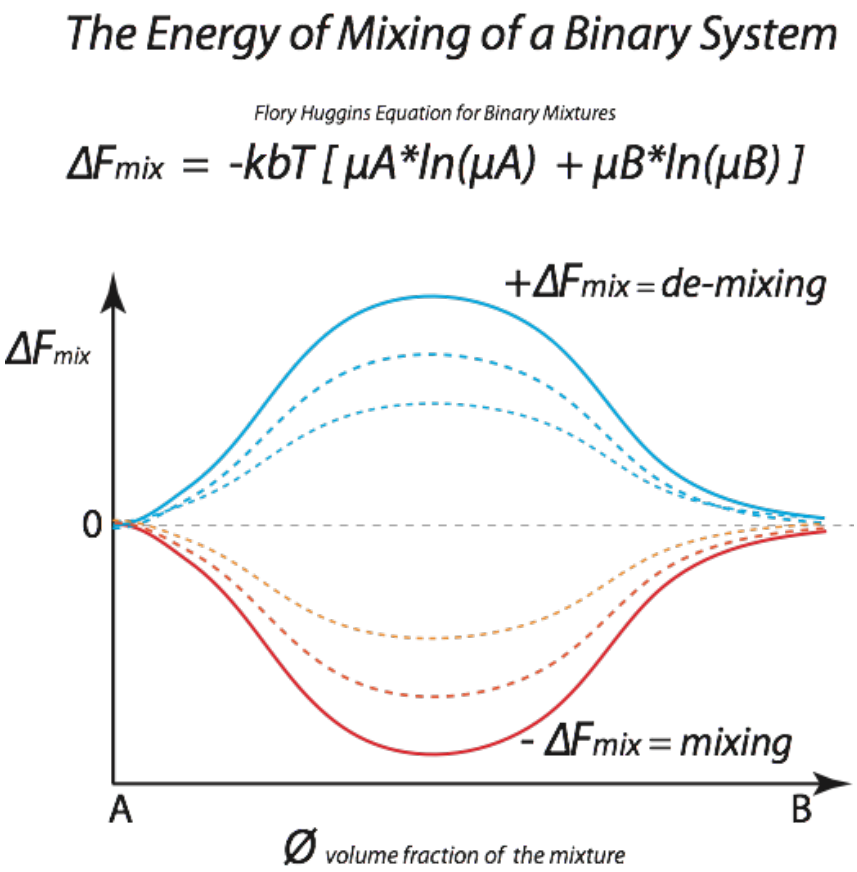


Figure I1. The Free Energy of a Binary Mixture of Component A and B = 1-B. The Free Energy ΔFmix (Flory - Huggins equation) describes the energy available to mix/de-mix a system of two liquid components. The component A and component B = (1-A) are represented in the x-axis in the form of their volume fractions μA and μB equation above as the respective chemical potentials. The red line represents the case where Temperature favors the mixing of the two components into one phase. Conversely the blue line represents the condition where Temperature favors the de-mixing of the two components into two phases.

In active phase separations for instance, the cytoplasmic mixture of proteins, is triggered to de-mix via the consumption of a source of energy, with higher orders of magnitude in (kBT) for which in vivo organelles such as centrosomes, it is necessary to lower the energy of mixing (ΔFmix) of the system creating via the utilization of ATP creating strong interactions between molecules with new conformational changes involved that would not be possible otherwise (Figure I2 A).

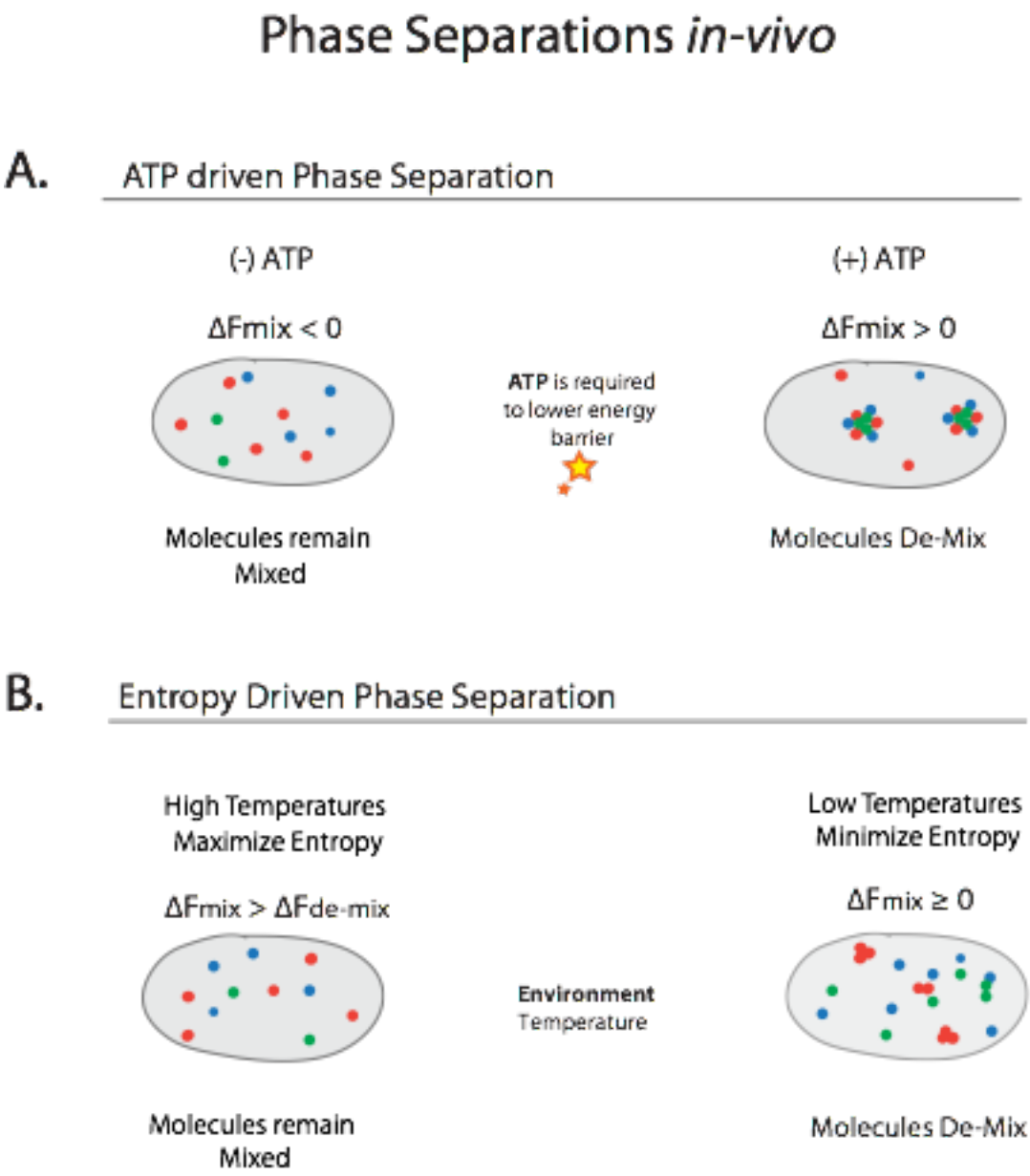


Figure I2. Phase Separations *in vivo* can occur via ATP consumption or driven by entropy. The energy of mixing in a liquid phase separation ΔFmix is determined by the competition between the entropy and the enthalpy of the mixture (Equation I1). A. In active Liquid Phase Separations, ATP fuels the reaction to form phase separated compartments as a source of energy to overcome ΔSmix to phase separate different components out of solution that would not come together by themselves. On the other hand in B. A Entropy Driven Liquid Phase Separation is mainly controlled by state variables such as temperature, pressure or change in volume fraction (concentration) allowing weak intermolecular interactions to drive the phase separation into de-mixed states forming membrane less compartments.

In contrast, in a entropy driven phase separation, molecules that have the tendency to mix at a certain temperature, would de-mix at another temperature granting the entropy of mixing (+ΔFmix) most of the main contribution of the phase separation because it screen for the energy of interaction between molecules until

they will phase separate. This phenomena occurs, just slightly above the thermal energy scale (kBT) due the presence of weak intermolecular interactions letting temperature to control the (ΔFmix) which becomes positive. Temperature alone can eventually drive the system at the boundaries between a mixed homogeneous mixture and a de-mixed state (Figure I2 B).

This means that weak molecular interactions such as electrostatic forces, drive the formation of a phase separation poised by temperature. And let the entropy to control whether the components of a mixture will phase separate.

In living cells, cytoplasmic components may phase separate in a concentration dependent manner as shown for the size scaling in the interior of cells and nucleoli dominated by a phase separation (Haataja and Brangwynne 2014), or via active energy consuming processes bringing soluble components together that would not come close otherwise from a cytoplasmic soluble state with the influence of ATP for their assembly such as centrosomes (Wueseke *et al.* 2012). However it is still unknown whether a liquid phase separation driven by temperature can indeed control the formation of any phase in the cytoplasm of living cells.

2.3. P GRANULES RESEMBLE LIQUID-LIKE PROPERTIES

The description of P granules as asymmetrically segregated cytoplasmic compartments was first reported using immunofluorescence of a set of proteins and in situ hybridization of RNA probes in *C. elegans*. P granules were distributed asymmetrically throughout the cytoplasm of fertilized embryos and being segregated towards the germ line of the nematode at the posterior side of the embryo (Strome and Wood 1982). The asymmetric segregation of P granules during early embryogenesis coincide with the asymmetric partitioning of RNA - protein maternal determinants known as polar bodies described in other species such as *Drosophila sp* (Mahowald 1962), in *Ciona intestinalis* (Shirae-Kurabayashi *et al.* 2006), where their constituent proteins *oskar* (Kim-Ha *et al.* 1991), *vasa* (Thomson *et al.* 2008), *piwi* (Yajima *et al.* 2014) and tudor (Boswell 1985;Vourekas

et al. 2010) protein family members accumulate in the germ cells of these organisms. The remarkable distribution of P granules and Polar bodies is towards the posterior side of the embryos where the germ line cells will differentiate and form the germ lineage. In principle the asymmetric distribution of these RNA - granules was thought to happen by the action of microtubules, as a purely mechanical phenomena in *C. elegans* and *Drosophila. sp* (Mahowald 1962;Hyman and White 1987;Gallo *et al.* 2008). AndIn the particular case of the nematode *C. elegans*, this process was hypothesized to occur by the pushing forces generated by microtubules towards P granules pushing them towards the posterior side of the P0 cell embryo during mitosis (Hyman and White 1987). However more precise descriptions on their asymmetric formation and distribution of P granules are necessary to understand the role of P granules as maternal determinants that differentiate the germ line from the soma; in special regard the process that partition the cytoplasmic maternal determinants into granular structures their formation and segregation of their components .

Traditionally P granules had been described as “punctae” or “granules”. However, more recently, P granules have gained an additional level of detail in their physical description as liquid-like compartments that resemble the physical properties of liquid droplets. As liquid - like droplets, P granules flow and shear like honey or glycerol in the cytoplasm whose state is precisely regulated across the antero posterior axis of the embryo in a process called; controlled dissolution/condensation liquid phase separation (Brangwynne *et al.* 2009b). Dominated by the cytoplasmic concentration of their components in supersaturation of P granule material, that in turn would induce their condensation into droplets, from the cytoplasm in a similar way as water vapor condenses when the air is very humid on a glass slide.

In contrast to centrosomes and nucleoli, P granules have a wide nucleation distribution all over the cytosol of a developing embryo before Polarity Onset (POn). Their localization is spatio-temporally regulated and segregated towards the posterior side of the P0 cell during the first cell division. In advanced embryonic stages, P granules sit on the surface of the nucleus in the P lineage

where they interact with the nuclear pores via hydrophobic interactions (Updike *et al.* 2011).

On the nuclear surface P granules exhibit liquid-like features such as wetting, shearing induced by external mechanical deformation, dripping, and fast exchange of P granule content with the cytoplasmic soluble material (Brangwynne *et al.* 2009b).

One of the most striking characteristics of liquid P granules is their cytoplasmic spatio-temporal regulation. During the cell cycle it accomplishes the dissolution at the anterior and P granule droplets formation and growth towards the posterior side of the embryo. This process can be achieved by the cell via a controlled dissolution/condensation (Brangwynne *et al.* 2009b) and is limited and regulated by the physical phenomena called Ostwald Ripening.

As liquids P granules condense and grow following Ostwald Ripening. This is the process in which an out of equilibrium emulsion (cytoplasm with droplets) where many droplets co-exist, can grow in size and liquid phase separate in two ways i) by accumulating components in solution at the droplet inner phase (RNA- protein) or ii) by growing at expenses of other droplet by the fusion with smaller droplets after a certain amount of time until two fully separated phases can be observed. In the case of P granules in *C. elegans* this process is observed during the first asymmetric cell division and is spatiotemporally controlled by other cytoplasmic factors such as MEX-5/6 as has been further hypothesized and shown in preliminary data in our lab (Saha, Hoege & Weber unpublished data).

The biochemistry of P granule formation is tightly linked with the enzymatic activity of the PAR (abnormal embryonic PARtitioning of the cytoplasm) proteins. During the first steps of polarity in the *C. elegans* embryo, PAR soluble cytoplasmic proteins localize from the cytoplasm to the cortex by advective flows (Goehring *et al.* 2011) in turn setting the anterior-posterior axis of the embryo. PAR-2, PAR-1 and LGL-1 form the posterior PAR complex (Hoege *et al.* 2010) and PAR-3, PAR-6 and PKC-3 form the anterior PAR complex. Together these two domains provide differential chemical identities to the cortex of the anterior and

posterior sides of the embryo. This differentiation, in turn, is crucial to create different cytoplasmic environments downstream, priming the spatiotemporal organization of the cytoplasm via the generation of cytoplasmic gradients, such as MEX-5 and MEX-6 to the anterior side of the embryo via PAR-1 and PAR-4 dependent phosphorylation (Tenlen *et al.* 2008).

P granules segregate towards the posterior axis of the embryo, in anti-correlation with the formation of the MEX-5/6 gradient which is enriched towards the anterior side of the embryo (Wu *et al.* 2015b). At the same time, the establishment of MEX-5/6 gradients serve to control the formation of an opposite gradient that lies towards the posterior side of the egg such as the PIE-1 gradient (Wu *et al.* 2015b) in a process that takes around 15-19 min during the end of Anaphase to define two cytoplasmic environments with different molecular and physical identities.

P granules, by differentially segregating towards the posterior side of the developing embryo, bring with them maternally provided RNA that determines the cell fate of the germ line.

The role of P granules as RNA carriers, RNA processing centers and germ line determinants is a highly discussed topic in *C. elegans* germ line development. Studies have shown that the existence of P granules as droplets confer identity to the germ lineage (Strome and Updike 2015). P granules are dynamic maternally segregated droplets that interact with multiple components in the cytoplasm (Beshore *et al.* 2011;Voronina *et al.* 2012;Wang *et al.* 2014). This shows that their role could be associated with the formation of liquid compartments that, throughout their maturation, accomplish a plethora of transcriptional activities within the P granule phase (Sengupta *et al.* 2013;Campbell and Updike 2015) This includes the tuning of physical properties induced by RNA binding such as viscosity and surface tension (Elbaum-Garfinkle *et al.* 2015) or their change into solid like states (Hubstenberger et al 2013). Many of the described P granule functions have been linked with RNA processing or RNA binding activities (Orsborn *et al.* 2007;Taylor *et al.* 2015), placing P granules as highly dynamic compartments with liquid-like properties whose function might be tightly linked

with their liquid-like state. Although the liquid like state of P granules remain unstudied in terms of P granule regulation and their physiological relevance

2.4. PHASE CHANGES AND THE CELL CYCLE

As previously explained, the cytoplasm of living cells accomplishes for a set of different cytosolic phases and compartments that are actively regulated during the cell cycle. In general, cell biology consider that these compartments are tightly regulated by chemical reactions, hence driving a timely formation of cytoplasmatic structures by dissipating energy in form of ATP. The process is driven by the transduction of phosphate groups via ATP hydrolisis keeping the pace of several other reactions that accomplish for an active cell. Regardless of the biochemical complexity of the cell, the thermodynamics of its biochemical reactions in vivo remain as an unexplored phenomena with special consideration under extreme environmental conditions, where entropy could disrupt or break the specificity among biochemical reactions and at the same time play an important role in the formation of or destruction of compartments.

A way to better understand the thermodynamics of the cytoplasm is to understand how physiological processes change, upon shift in the temperature at which a cell normally form its organelles. Experimental approaches like these had been made in our lab by Maria Begasse, utilizing two species of nematodes that are adapted at two different temperatures: *Caenorhabditis elegans (18°C)* and *Caenorhabditis briggsae (25°C).* The idea is to compare the thermal response of the cell cycle rate between both nematodes whose temperature ranges of maximal fertility are different (Tfer = 27 for *C. elegans*; Tfer = 30 for *C. briggsae*). The findings reveal that these nematodes have in essence a species specific thermal response i) a physiological thermal response where the cell cycle increases exponentially up until a temperature where the cell cycle breaks down (T*) ii) the thermal limit where only the fertility of the worm is compromised but not its survival or (Tfer). The findings indicate that the cell cycle rate in both species scales with

temperature following an Arrhenius-like relationship throughout the cell cycle progression. Furthermore when temperatures were at the limit of the fertile thermal range (Tfer) for the two nematodes, the cell cycle rate abruptly breaks down by dramatically slowing down the cell cycle rate of the worms, loosing synchrony between intracellular processes (Begasse *et al.* 2015b). These observations suggest that some type of biochemically active "master regulator" of the cell cycle would suffer the effects of high temperature and lose control over the reactions involved in the progression of cell division. One interpretation if this is that the breakdown is caused by phase change or phase transition in one component of the cell cycle that is essential for its progression. Furthermore, it could be predicted that changes in this mechanisms account for the difference between these two species?

In general terms a phase transition occurs as a global feature of the thermodynamic system, for instance (quoted from Kumamoto):

-*The conversion of water to ice is a simple example of a phase change. The essential feature to recognize is that the activity of water in water, and the activity of ice in ice is constant and independent of the amount in each state* -(Kumamoto *et al.* 1971).

This leads us to consider that a phase change between liquid water and ice state happens throughout all the system at a single temperature at once. This becomes obvious to our perception when water freeze and is evidenced as a strong discontinuity in the temperature dependence of the physical change from liquid - ice and ice - liquid. That in the case of the cytoplasm, the amount of components place the system far away from a pure phase transition as depicted for water and ice, however due the multifactorial nature of the cytoplasm it is possible that a fraction of the cytosolic components would undergo a liquid phase transition driven by temperature in the same way that occurs during the water-ice transition.

The implications of this is that if the experiments of Beggasse *et al.* suggest that a drop off in the cell cycle rates indicate that there is a cytoplasmic phase transition as suggested by others referring to an Arrhenius drop off (Biosca *et al.*

1983), there should be a direct observable macroscopic cytoplasmic change in the nematode's embryo. However this observation does not have a clear scientific description nor thermodynamic explanation to date.

Further experiments from Begasse et al. have focused in describing the type of Arrhenius relationship and the thermal window where embryos are more susceptible to suffer a drop off in fertility induced by temperature. The authors found that this window occurs during the first asymmetric cell division in P0 cell. Several factors are temperature sensitive in the worm, specially those related with the cell cycle progression (Hirsh 1979), however those related with the germ line are of special interest for this study considering that perhaps a phase transition could occur specifically in the germ line where liquid P granules and other cytosolic compartments are formed.

The cytosol of the germ line has the special property of containing liquid-like compartments such as P granules. The liquid nature of P granules is in particular an interesting feature of P granules with regards to temperature dependence, due their interfacial properties with the cytoplasm, fast exchange of material and viscosity. In addition it has been previously reported that PGL-1 and PGL-3 are constitutive P granule components associated with nematode sterility at high temperatures (Kawasaki *et al.* 1998a). As previously described PGL-1 has been shown to have striking liquid-like properties at short time-scales throughout the nematode's cell cycle, and are thought to be actively controlled via the influence of cytoplasmic factors (Wang *et al.* 2014;Wu *et al.* 2015a). P granule are therefore excellent candidates for the cytoplasmic components that undergo a phase transition that affects fertility. This raises the question of whether P granules undergo a cytoplasmic liquid phase separation, driven by temperature.

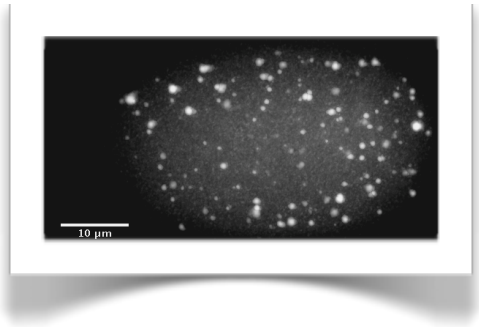
In this research project, I have focused to find a thermodynamical description for P granule formation as a liquid phase separation driven by temperature. And explore their biochemical dependence of their formation. Finding that there is indeed a correlation between P granules as liquid droplets and the worm's fertility, a liquid phase separation that form droplets and temperature which drives

the phase separation and define the fertile thermal range of *Caenorhabditis elegans*.

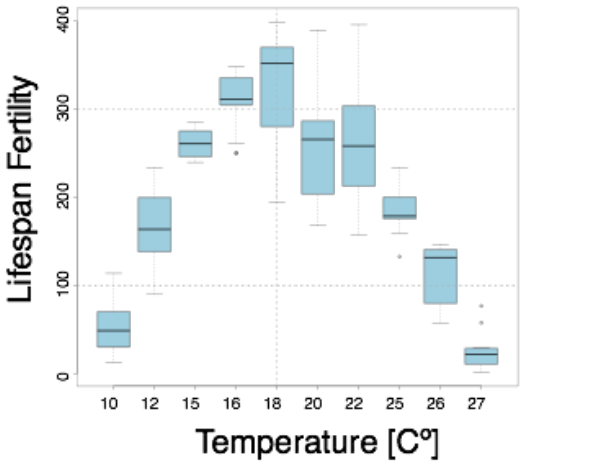
3. Aim

Is there a link between P granule Liquid Phase Separation and the Fertile Thermal Range of Caenorhabditis elegans?

P granules in a P0 cell embryo before Polarity Onset



Temperature Fertility Range of C elegans



4. Methods

4.1. STRAINS

The strains used in this study were ordered from the Caenorhabditis Genetics Center (CGC) (Table 1). Given that temperature is an important factor in our study, strains were reared on a case by case manner at 18ºC or 25ºC, depending on the caveats regarding gene silencing and fluorescence at low temperatures. Nematodes were grown in NGM agar medium with *E. coli* (OP50) at 18ºC and 25ºC as explained above.

Strain	Genotype	Reference	Temperature
JH2019	unc-119(ed3) III; axIs1466.	(Gallo *et al.* 2010b)	maintained at 18ºC
JH2835	pptr-1(tm3103) V; axIs1504	(Gallo *et al.* 2010b)	maintained at 25ºC
JH2840	unc-119(ed3) III; axIs????.	(Gallo *et al.* 2010b)	maintain at 18ºC
JH2841	pptr-1(tm3103) V;axIs1522	(Gallo *et al.* 2010b)	maintained at 18ºC
JH2787	pptr-1(tm3103) V	(Gallo *et al.* 2010b)	maintained at 18ºC
JH2329	unc-119(ed3) III; axIs1488	(Gallo *et al.* 2010b)	maintained at 25ºC
JH2330	unc-119(ed3) III; axIs1488; axIs????	(Gallo *et al.* 2010b)	maintained at 25ºC
JH2836	unc-119(ed3) III; axIs????	(Gallo *et al.* 2010b)	maintained at 18ºC
TH206	unc-119(ed3) III; axIs1498	(Brangwynne *et al.* 2009b)	Maintained at 18ºC

Table 1. Strains used for temperature experiments were reared, tested and screened for constant and stable fluorescence at different temperatures.

4.2. TEMPERATURE CONTROL

The main focus of this work involved performing time lapse microscopy on embryos while maintaining precise temperature control of the sample. Initially, the traditional agar pad method for mounting and imaging embryos was used. The agar is an isotonic mixture of agar and phosphate buffer with 0.2% glucose in which the embryo is immersed, protecting it from dehydration and physical compression from the cover-slide. The pad is made from a drop of agar melted at 60°C, that is subsequently cooled down and sandwiched between two glass slides that are then squashed together in order to create the agar pad of agar that is approximately 100 to 110 µm thick. Embryos dissected out of the gravid hermaphrodites and placed with a mouth pipette on the agar pad. Embryos mounted in this way sit on top of the agar pad with a cover slide on top, and are then mounted on the microscope stage.

The method mentioned before was not useful for quantitative temperature controlled experiments, due to irregular temperature dissipation across the agar pad. In addition, upon temperature upshifts the agar pad experienced thermal deformations; inducing drifts of more than 20 µm in all axis, making it difficulty to acquire fast, high-resolution images of the whole embryo under a confocal microscope or other kind of microscope. For this precise measurement a technical improvement had to be made to control the temperature of the sample chamber where the embryo sits.

4.2.1. HEATING/COOLING SETUP DEVELOPMENT AND MICROSCOPE STAGE

In most worm-rooms temperature is around 16ºC to 24ºC. However during the course of a time-lapse experiment , and several hours of work at the stereo microscope, heat from microscope electronics increases the temperature at the mounting station up to 30ºC. Embryos exposed at these temperatures gave inconclusive results, since the local temperature was assumed to be within 24ºC and 30ºC range difference and uncontrolled experiments were giving inconclusive results.

To stabilize the conditions, a heat sink for the confocal and dissection stereomicroscope were designed and adapted to both microscope stages in order to maintain temperatures below 20ºC. The heating/cooling setup would dissipate away excess of heat from the sample mounting area, thereby allowing the precise control of temperatures at the imaging site and on the dissection scope. In order to efficiently absorb and conduct latent heat away from the chamber, the heat sink was made of a block of aluminum with etched channels inside, that conduct water at 10ºC at a high flow rate ~ 1L/min.

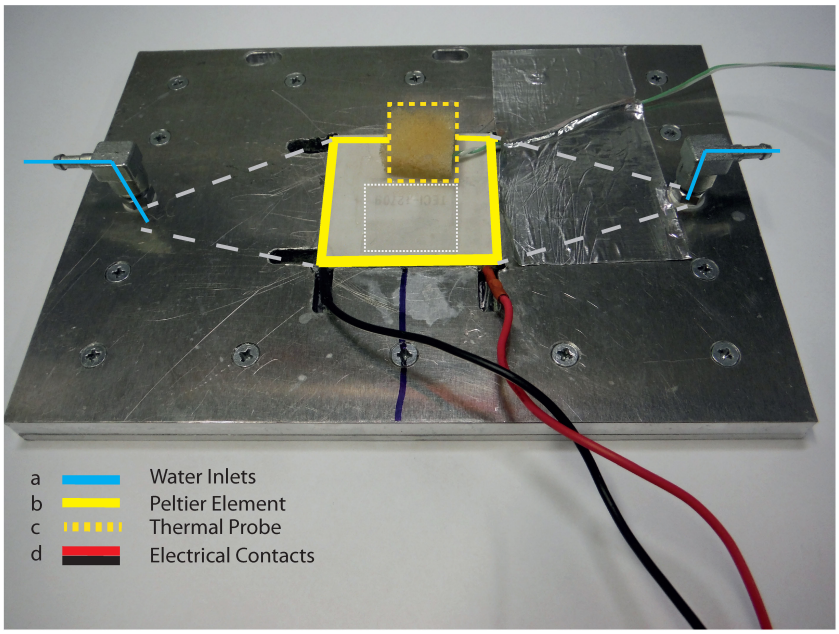


Figure M1. Heating Cooling Device with Sample Holder. Device used to provide thermal quenches to nematode embryos, gonads and *in vitro* experiments mixtures.

4.2.2. CONFOCAL SAMPLE HOLDER AND HEATING/COOLING DEVICE

The aim of developing a temperature controlled device is to precisely quench temperature rapidly while performing live time-lapse microscopy under a confocal spinning disc microscope. For this, a peltier element was adapted as a heating/cooling component of the heating setup and was designed to operate on top of a heat sink made of aluminium. The peltier element offers a surface where the sample can be placed, with dimensions of 18 x 18 mm. Here, temperature is constant over long time experiments. In order to provide a clean surface for the sample to sit on the peltier element, this was covered with a glass slide and treated with an anti-wetting agent to provide a smooth, regular surface at the center of the peltier element (Figure M1).

Additionally, in a separate location from the sample holder, a thermal probe is placed at approximately the same distance as the sample would be from the sample holder, creating a second chamber with similar dimensions where the probe can record temperature. The probe is connected to a logger device (National Instruments) and temperature data is recorded every second. Variations in temperature over long temperature exposures were of 0.1°C every hour with a

maximum duration of 30 seconds each, demonstrating the stability of the heat sink.

4.2.3. SAMPLE PREPARATION

In order to place the sample on top of the heating/cooling device, a sample chamber was necessary to keep the sample isolated from the environment and to avoid desiccation. For *in vitro* experiments a sample holder was also necessary to contain the sample volume tested in every experiment.

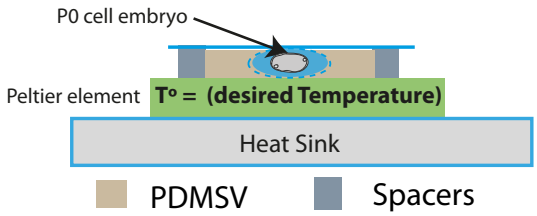


Figure M2. Sample Holder. The sample holder is designed to isolate the sample from its surroundings and allow long term imaging. In grey, sample is separated from the peltier surface with 100 µm distance. In brown, PDMSV acts as a milieu with low thermal expansion, where the sample (blue dotted line) sit and droplets or embryos can be imaged.

The sample is prepared on a cover-slide sealed by silicon grease acted as a malleable boundary that kept the sample contained and at the same time served as a spacer for the sample holder (Figure M2). Once the sample was positioned inside of the sample holder, it was turned up side down on top of the peltier element, creating a chamber containing the sample.

To keep the sample volume fixed in the sample chamber, a cover-slide was prepared using a mixture of grease and silicon beads which acted as spacers between the sample holder and the ceiling of the sample chamber. Spacer beads had a 30 µm diameter (Sigma-Aldrich) and no fluorescence.

The beads created a constant volume in the chamber and the silicon grease protect the sample from desiccation, when different temperatures were applied.

4.2.4. TEMPERATURE OF THE MICROSCOPE OBJECTIVE

To maintain a constant temperature on the z-axis two collars were installed around a 63X glycerol immersion objective. One collar is electrically controlled to heat up the lens using a resistor wrapped around the objective, the other collar was a tube that surrounds the objective which conducted cold water around it. The tuning between both heating and cooling collars, gave the lens a dynamic range of use that regularly matched the temperature cycles that the temperature chamber provided to the sample to appropriately image the embryo at the desired thermal conditions. Both collars were synchronized in a way that at 18ºC would reduce the thermal difference on the z-axis to less than 1ºC over a 500µm distance.

4.3. *IN VIVO* ASSAYS

Caenorhabditis elegans worms were dissected on a temperature controlled stereo microscope deck kept at 18ºC. This temperature matched the temperature at which the nematode was grown. P0 Embryos at the stage before Pronuclear Migration were extruded from the gonad by dissection of gravid hermaphrodites and placed in the sample holder as depicted in Figure M1. Subsequently the sample holder with the nematode embryos was turned upside down and placed on top of the peltier element under the confocal microscope. A 63X objective with a 1.3 N.A was used with lateral resolution of 107 nm per pixel, and axial resolution of 800 nm in z. A stack with dimensions of 288 pixel width and 546 pixel height, was set in order to capture a single whole embryo volume using a z-step of 300 nm with a piezo electric motor attached to the base of the objective.

Camera noise and sensitivity of the camera chip was evaluated and set as a minimum of 450 in arbitrary intensity units at different laser powers, canceling out any background fluorescence from the buffer or other elements used in the assay. The laser power used for imaging the embryos was set as 200 µW, which gave a maximum value of GFP intensity across all the samples of 17550 A.U. in a dynamic

range of a 16-bit image corresponding to the maximum intensity of the brightest P granule in very stack. The cytoplasm was analyzed in the same manner, having an average fluorescence intensity of 850 +/- (300) A.U..

Images were processed using FIJI (Pietzsch *et al.* 2012;Schindelin *et al.* 2012;Schneider *et al.* 2012;Schindelin *et al.* 2015), from time-lapse recordings of embryos expressing PGL-3::GFP. and processed to extract information about fluorescence intensity values and P granule Volume. Macros were used to normalize and separate the P granules fluorescence from the cytoplasmic fluorescence. P granules were detected using a threshold of 1200 A.U as a minimum value to consider as the minimum signal belonging to a P granule. Furthermore, in order to volumetrically detect P granules, the 3D counter (BOLTE and CORDELIÈRES 2006) was used and set to detect particles above 300 nm in size. Given that the intensities of P granules vary with size, it is not possible to rely on a single mean value of intensity as a proxy for single P granule size. In order to create a criteria to determine that a certain particle is a P granule, the variation coefficient of the mean intensity per droplet was used to distinguish droplets from random pixel correlations assuming that there is a variation from the mean of 10% in intensity units. In addition particles below 1200 A.U were left out of the threshold and detected volumes under 300 nm were also excluded by the particle definition criteria to filter out false positives. Data was generated using FIJI and tables were analyzed using R (TEAM 2010).

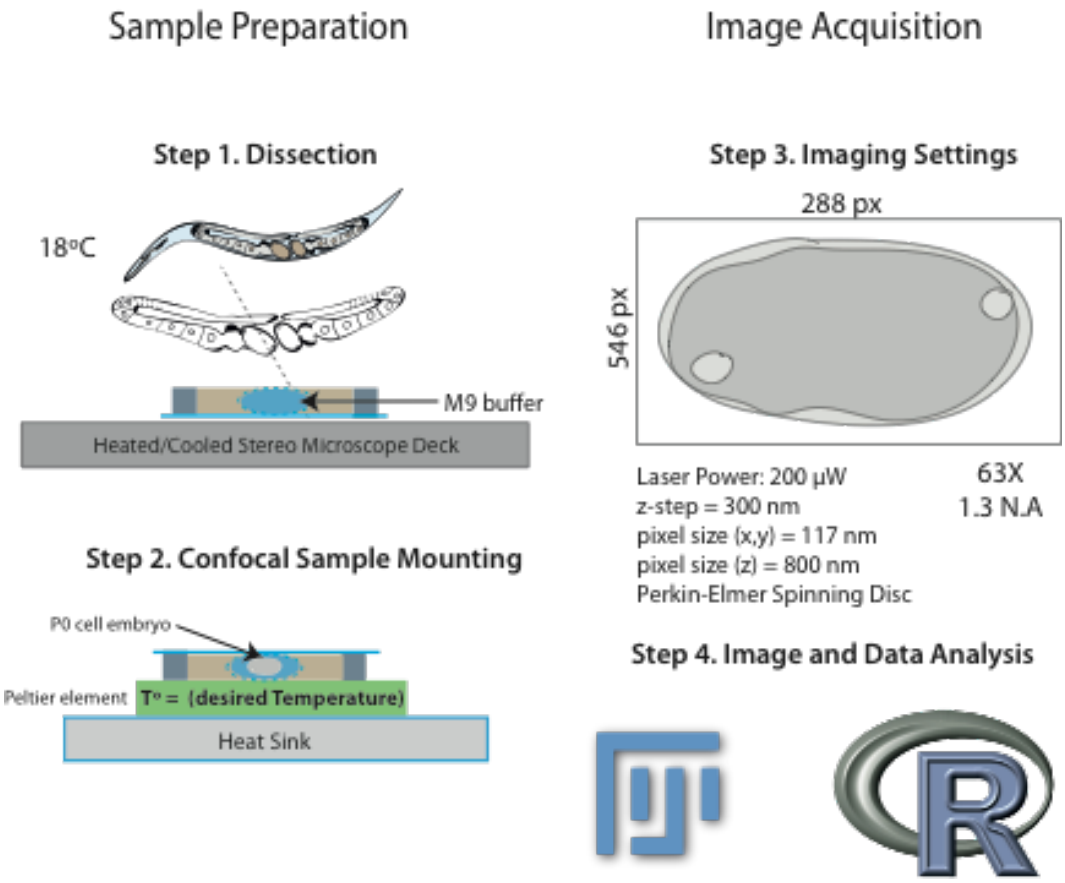


Figure M3. *In vivo* Sample Preparation and Image Acquisition. In Step 1, dissected embryos are placed in the temperature controlled area then imaged and analyzed using ImageJ and Rstudio Step 2,3.

4.4. *IN VITRO* ASSAY

To test if temperature can drive P granule-like compartments *in vitro,* PGL-3::GFP protein was purified and kindly provided by Saha (unpublished data). Protein was stored in buffer containing 300mM KCl at -80°C. PGL-3::GFP protein was assayed at 300 mM KCl as an initial homogeneous state where no droplets formed. Droplets were observed when triggered by a decrease in salt concentration. Droplets formed at concentrations of KCl lower that 250 mM. We decided to use a concentration of 150 mM as this matched physiological concentrations of KCl previously reported (Figure M4).

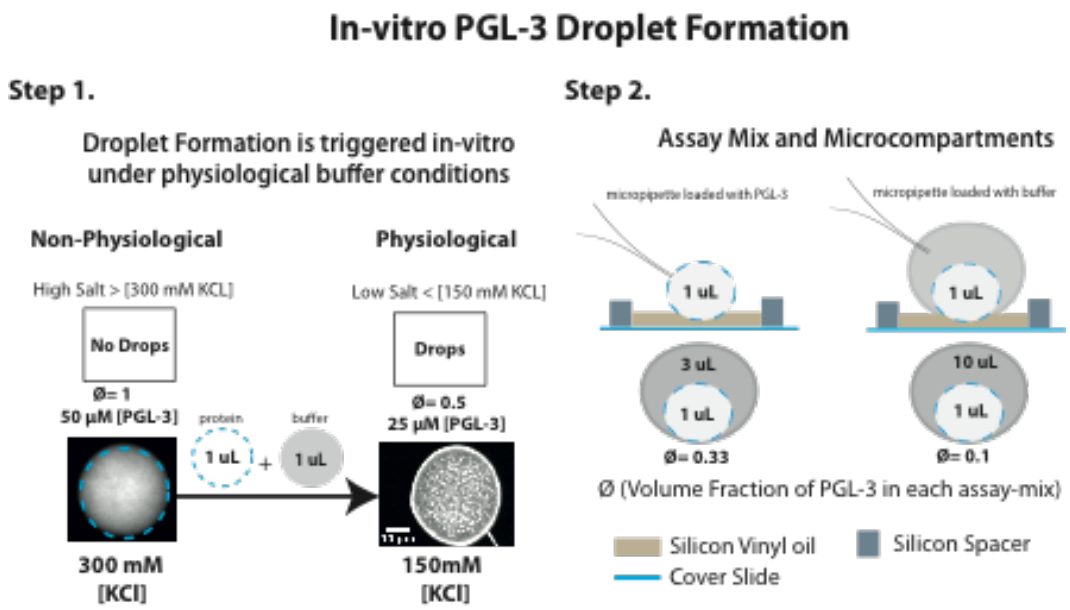


Figure M4. *In vitro* Droplet Formation. Droplets are triggered to form by lowering the concentration of KCl to 150 mM. Step 1, assay mix is formed i*n situ* in order to trigger the reaction all at once; concentrations are manipulated by changing the component volume fractions.

Solutions of different protein concentrations were made and assayed to evaluate the formation of droplets under the microscope, following the methods for sample mounting described previously.

PGL-3::GFP forms viscous droplets and has a strong tendency to sediment and stick to surfaces. In order to keep the protein isolated from glass surfaces, the formation of droplets was triggered at the sample chamber as depicted in Figure M5. This was done by injecting the protein in a matrix of liquid Poly Dimethyl Siloxane Vinyl terminated (PDMSV), as a liquid non-wettable milieu where PGL-3 protein could be contained. Initially PGL-3::GFP was in buffer with 300 mM KCl, then subsequently diluted to achieve 150 mM KCl and a protein concentration that triggered the formation of droplets at any PGL-3 volume fractions.

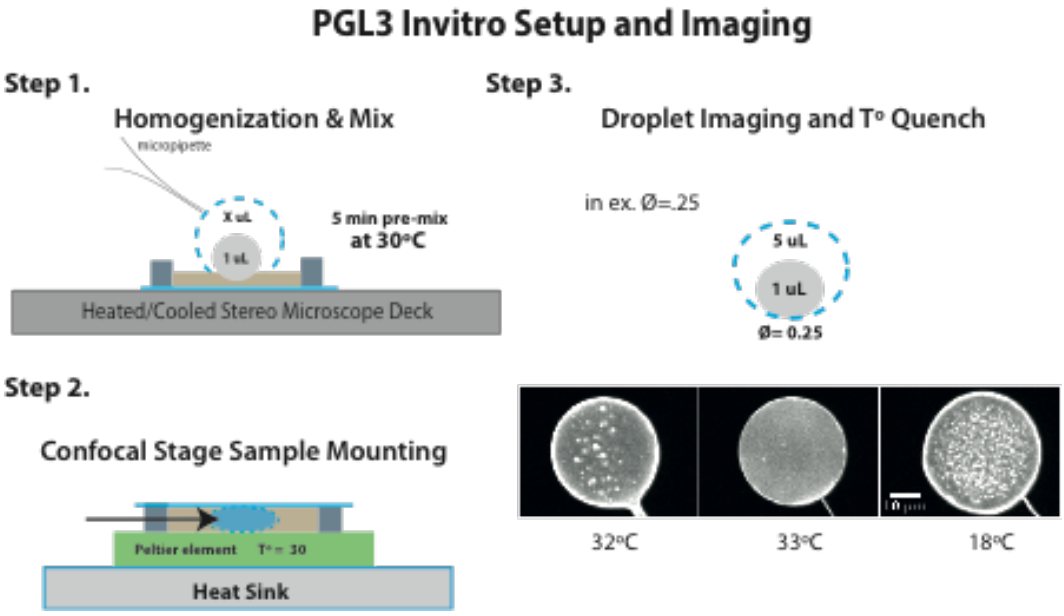


Figure M5. Droplets are triggered to form upon a temperature quench. After Homogenization & Mix Step 1, the assay is started under the microscope at a temperature where droplets are mix and a T quench is made Step 3.

As mentioned before, different protein concentrations were achieved adjusting the volume of the reaction buffer while keeping the amount of added protein at 1µL per assay. Further adjustments were made in order to keep the amount of KCl in the physiological levels at around 150 mM, as required for each assay. This condition was further standardized in Figure M4, step 2.

During the mixing process, it was observed that as the solution began to mix, droplets were formed in the PDMSV compartment depicting regions with high amount of droplets and other regions with low amounts of droplets, this undesired effect was due the inhomogeneous nature of the mixing process after injecting PGL-3::GFP in high salt buffer. This phenomena is not suitable to perform a thermal quench, because both fractions were not in fully mixed at the moment of the thermal quench This meant that the concentration of PGL-3::GFP was irregular over the sample chamber. The solution this problem is not to trigger droplet formation during the mixing of the sample. Instead, the protein was homogenized at 32°C and further mixed on a temperature controlled stereo microscope deck at 30°C, keeping the protein in a mixed phase before any temperature quench could be applied (Figure M5). After the homogenization and mixing steps were performed, the sample holder depicted in Figure M5 step 1,

was turned upside down and placed on top of the peltier element, creating a wafer between the sample and the heating device, where the mixed protein and buffer preparation were ready to be imaged at different temperatures (Figure M5 steps 2 and 3). The results were collected in tiff images and assessed visually in binary form (droplet, no droplets) to construct a binary phase diagram of the assay.

5. Results

5.2. TEMPERATURE AND P GRANULE PHASE SEPARATION

The cytoplasm is a crowded environment with a density of protein of 100 mg/mL (Kühn *et al.* 2011), that provides the elements for most of the cell chemical activity in a specific manner, by modifying the chemical potential of its constituents via postranslational modification and by tuning other type of molecular interactions such as electrostatic forces that can drive the formation of compartments and their spatial regulation as is the case for the partitioning of P granules.

Here the thermodynamics of cytoplasmic partitioning of PGL granules is studied. Results indicate that PGL-1 and PGL-3 P granule constituents can be driven to form droplets driven by temperature following a liquid phase separation of P granule cytoplasmic material. How P granules are affected by chemical regulators, in this case PPTR-1 phosphatase is also explored. In addition, and given the importance of P granules as germ line specific compartments, the thermodynamic behavior of P granules is linked with the thermal fertile range of *Caenorhabditis elegans* by finding that their thermal range correlates with the temperature at which P granules mix with the cytoplasm.

5.3. P GRANULES ARE TEMPERATURE SENSITIVE COMPARTMENTS

In order to understand the thermodynamic behavior of a liquid phase separation it is crucial to understand its temperature dependence. To address the question of whether or not P granules are temperature sensitive liquid compartments, a

high precision temperature controlled chamber was built to provide fast precise temperature quenches to P0 embryos during Polarity Onset (PO).

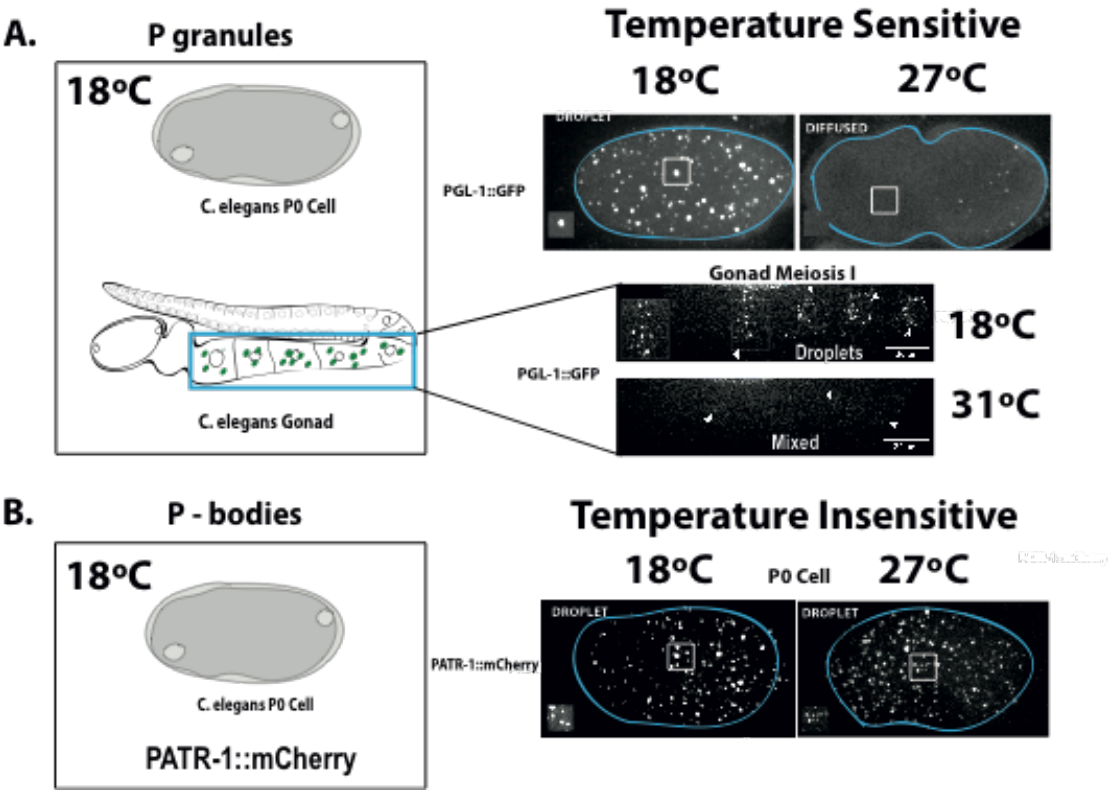


Figure 1. P granules are temperature sensitive membrane-less compartments. P granules dissolve in the cytoplasmic phase at 27ºC in the embryo and the gonads of the *C. elegans* nematode A. whereas P bodies remain stable at either thermal regimes during P0 B..

Embryos in P0 cell stage from the PGL-1::GFP strain (TH206) were dissected and exposed at two chosen temperature regimes according to the nematode maximum fertility temperature Tfer=18ºC and minimum fertility temperature Tmfer=28ºC characterized by Beggasse et al (2015). Embryos were dissected out of the worm's gonad in a temperature controlled stage and imaged under an upright confocal microscope. If P granules behave like liquids that phase separate from the cytoplasm, it is expected that an increase in entropy would make P granules mix their contents into the cytoplasmic bulk. Two questions are addressed here; i) are P granules sensitive to temperature? ii) can we relate the mixing boundary of P granules with the limits of nematode fertility shown in Begasse *et al.*?

At temperatures between 10-24°C, there was robust formation of P granules and a relatively low level of PGL-1::GFP observed in the cytoplasm. Between 24-27°C there seemed to be a decrease in the number of granules. However, at 28 degrees there were only a few very small P granule-like spots of fluorescence and a corresponding increase in the cytoplasmic level of PGL-3::GFP. This showed that P granules fully mixed with the cytoplasm at 27°C (Figure 1.A). As a control PATR-1::mCherry was imaged. PATR-1 is a component of different than membrane-less compartment that is called a P-body and has similar characteristics to P granules. However, similar temperature experiments on PATR-1::mCherry showed that they had no thermal response at the same thermal regimes (Figure 1.B). This means that the tendency of PGL-3 containing P granules to demix at 27°C is a specific property of these droplets and is not a general effect of temperature on the cytoplasm.

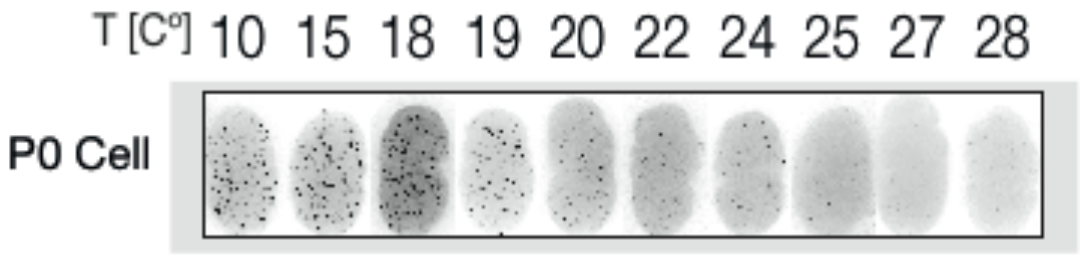


Figure 2. Embryos in Polarity Onset at different Temperatures. From Left to right 10 representative embryos were placed under the temperature controlled confocal microscope. Maximum projections of whole embryos (101 images per stack) show that P granules (in black) are present over a long temperature range 10 to 25ºC however P granules smoothly mix with the cytoplasm (grey) at different temperature regimes when exposed at different temperatures before Polarity Onset. It is observed how P granules mix with the cytoplasm passing from a droplet state towards a fully mixed state. At 27ºC some embryos still showed 1 to 5 small P granules; for this a +1ºC temperature control was performed (28ºC) to show that P granules are fully dissolved by temperature.

This striking result highlights the intriguing resemblance between the formation of P granules with a liquid-liquid phase separation in a complex and dense cytoplasm. Further experiments show that P granules not only have a strong temperature response at 28°C but their mixing range with the cytoplasm occurs

across a wide range of temperatures. This was further confirmed by following P granule fate at different cell cycle times (Figure 2) (missing figure)

Temperature is an important regulator of a liquid-liquid phase separation, controlling the entropy of de-mixing of P granule formation disrupting weak interactions between PGL-1/3 molecules that account for the formation of the liquid P granule droplets. This effect had been observed during the first asymmetric cell division in P0 cell stage (Figure 1), in the gonads, and oocytes (Figure 3). Based on this results two questions are relevant to study P granule formation.

1) What is the specific temperature at which P granules mix with the cytoplasmic phase?

2) Are these observations driven by an active chemical process?

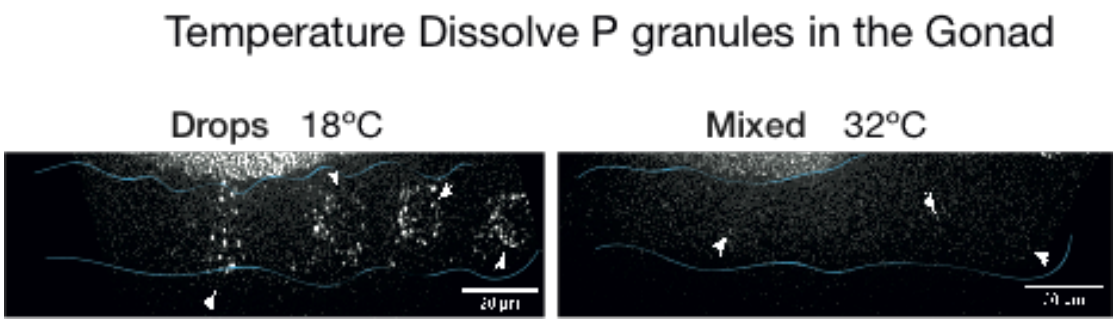


Figure 3. Temperature Controls Droplet Formation in the nematode's gonad. Upon a temperature quench from 18ºC to 32ºC, PGL-1::GFP droplets mix with the cytoplasm and re-condense once temperature is set back to 18ºC within 30 min.

5.4. P GRANULES MIX WITH THE CYTOPLASM AT 27ºC

To understand how P granules dissolve, it is important to quantify how much of the P granule volume is phase separated at each temperature (equation 1). Experiments were performed at different thermal regimes and the total volume of P granules at different temperatures was quantified, in order to estimate at what thermal regime P granules fully dissolve in the P0 cell during polarity onset and in the gonads of the embryo.

When the volume of the phase separated material (equation 1) is zero at a given temperature, P granules have been mixed with the cytoplasm with no droplets formed in the cytoplasm.

$$Vt = \frac{\sum_i^k Vi}{Ve}$$

Equation 1. Total Volume of Phase separated P granules per embryo (Vt). Where (i) is the ith P granule in the cytoplasm of an embryo and k is the total number of P granules per embryo. Ve stands for the Volume of a single embryo in µm³.

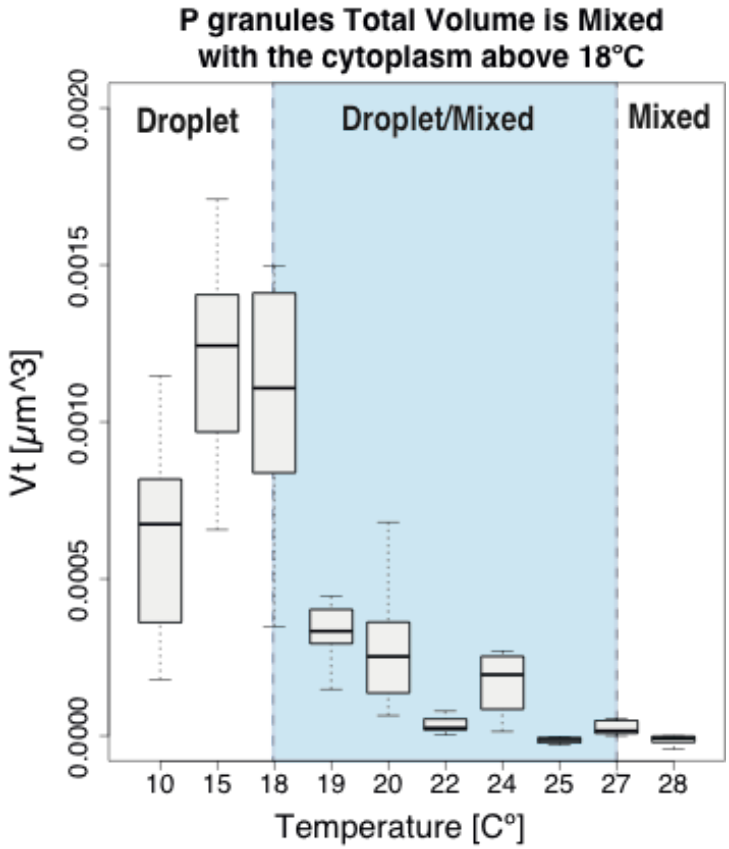


Figure 4. Volume Fraction of PGL-1::GFP granules Phase Separated from the Total Embryo Volume at Different Temperatures. PGL-1::GFP granule (Vt) is smoothly lost above 18ºC up to 28ºC where there is no phase separated PGL-1::GFP material.

The temperature where P granules fully mix together with the concentration of P granule components in the cytoplasm, describes the transition point where liquid

droplets are mixed. For PGL-1::GFP granules the P granule number (Np) per embryo, total phase separated P granule volume (Vt), and P granule total phase separated intensity (Ip) were measured at different temperatures (Figure 4).

When different temperature regimes were applied to the embryos in P0, it is shown that P granule total volume (Vt) do not have a single temperature where P granules pass from a de-mixed to a mixed state, even thought embryos were exposed some minutes longer to discard timing effects on the phase separation kinetics. P granule volume remain low from 20°C to 25°C however most of PGL-1::GFP material (Vt) sharply fell between 18°C and 19°C. Within this temperature span P granules maintain their highest phase separated volume loosing two thirds of the accumulated volume in comparison with 18°C (Figure 4).

The remaining P granule phase separated volume is lost across several temperatures from 19°C > T° < 26°C (Vt) reaching a full mixed state at temperatures above 27°C where (Vt) of the volume of the embryo during polarity onset (Figure 4).

At 27°C most of the P granules were dissolved, however small and faint pixels whose intensities were similar to the cytoplasmic background but were spatially correlated formed faint P granules, were also considered in the analysis. Their (Vt) further dropped off to even lesser volume values Vt << 0.0005 % at higher temperatures where a fully mixed state was achieved at 28°C.

When evaluating P granule numbers (Np) and volume (Vt), a similar trend seemed to show a massive initial dissolution of droplets followed by a broad dissolution ranging over 10°C (18°C to 28°C), suggesting that PGL-1::GFP leaves the droplet phase in great amounts after 18°C, but could maintain residual amounts in the droplet phase as temperature approaches 27°C. this hypothesis suggests that the multiplicity of components in the P granule phase could extending the range at which (Vt) falls at 18°C finally reaching a mixed state at 28°C from the total phase separated P granule material (Figure 4).

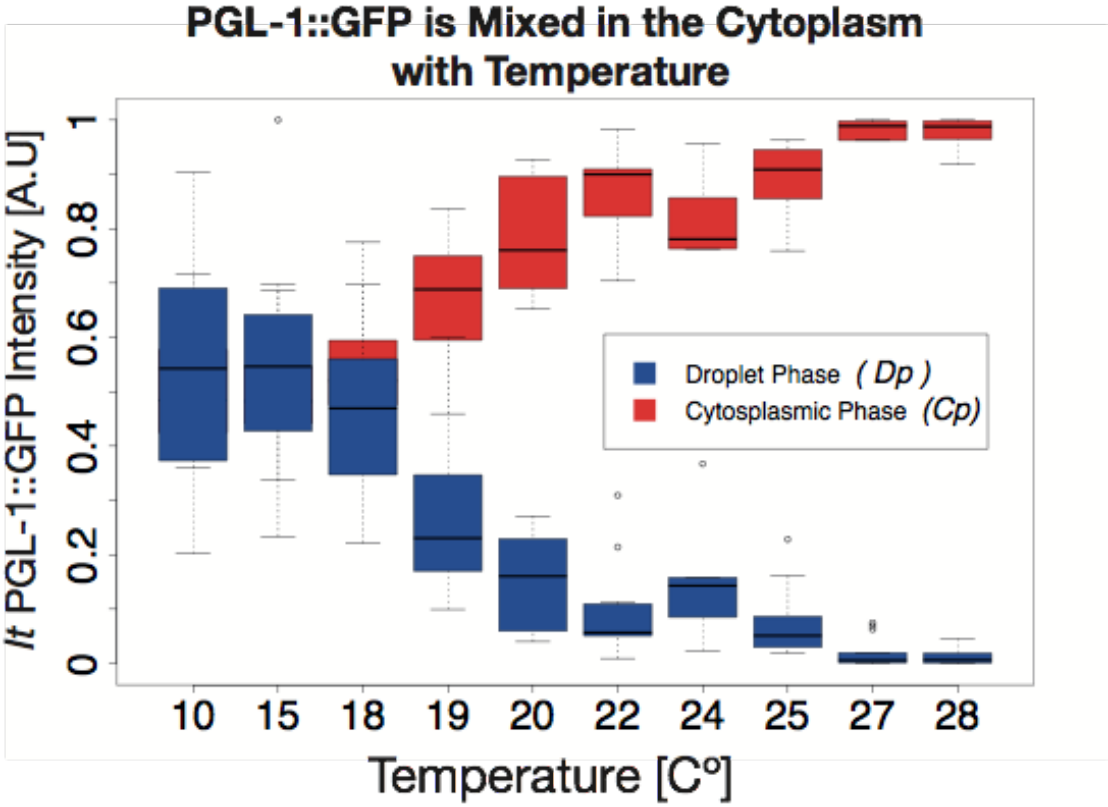


Figure 5. The cytoplasmic (Cp) and Droplet (Dp) content of PGL-1::GFP are shown inverse to each other as temperature increase. Total PGL-1::GFP intensity ØT=(Dp+Cp) do not have a significant loss of fluorescence at different temperature regimes, showing that droplets effectively become cytoplasmic after temperature increase.

$$Ør = (Øin - Øout)/Øt$$

Equation 2. The difference between the concentration in the droplet phase and the concentration in the cytoplasmic bulk Ør is accounted as the Order Parameter of the P granule Phase Separation. Note that when Ør = 0, the fraction Øin and Øout have reached equal parittions, accounting for 50% of the total PGL-1::GFP (*It*) in both Dp and Cp.

Further controls showed that there was no loss of fluorescence by bleaching or any unexpected increase in fluorescence from PGL-1::GFP induced by changes

in temperature. This was further analyzed by comparing the normalized droplet intensity fractions (Dp) and cytosolic intensity fractions (Cp) and were shown to be constant without a significant loss of total PGL-1::GFP at each temperature (Figure 5).

In order to determine whether PGL-1::GFP granule phase separation occurs as volume or punctae loss; different temperatures within the range 10°C to 27°C were compared, to see if there was a drop off in both P granule number, and P granule volume. This data was compared with the total droplet intensity (Dp) versus total cytoplasmic intensity (Cp) finding that a drop off in puncta, volume and intensity occurred within the same temperature ranges. However in order to better characterize whether P granule phase separation *in vivo,* the comparison between the concentration outside and in side of the droplet phase would better serve as an order parameter. The difference in P granule concentration inside of the droplet phase (Ø_in) minus the total concentration of PGL-1 in in the cytoplasmic phase is Ør as the order parameter of the phase separation as show in Equation 2.

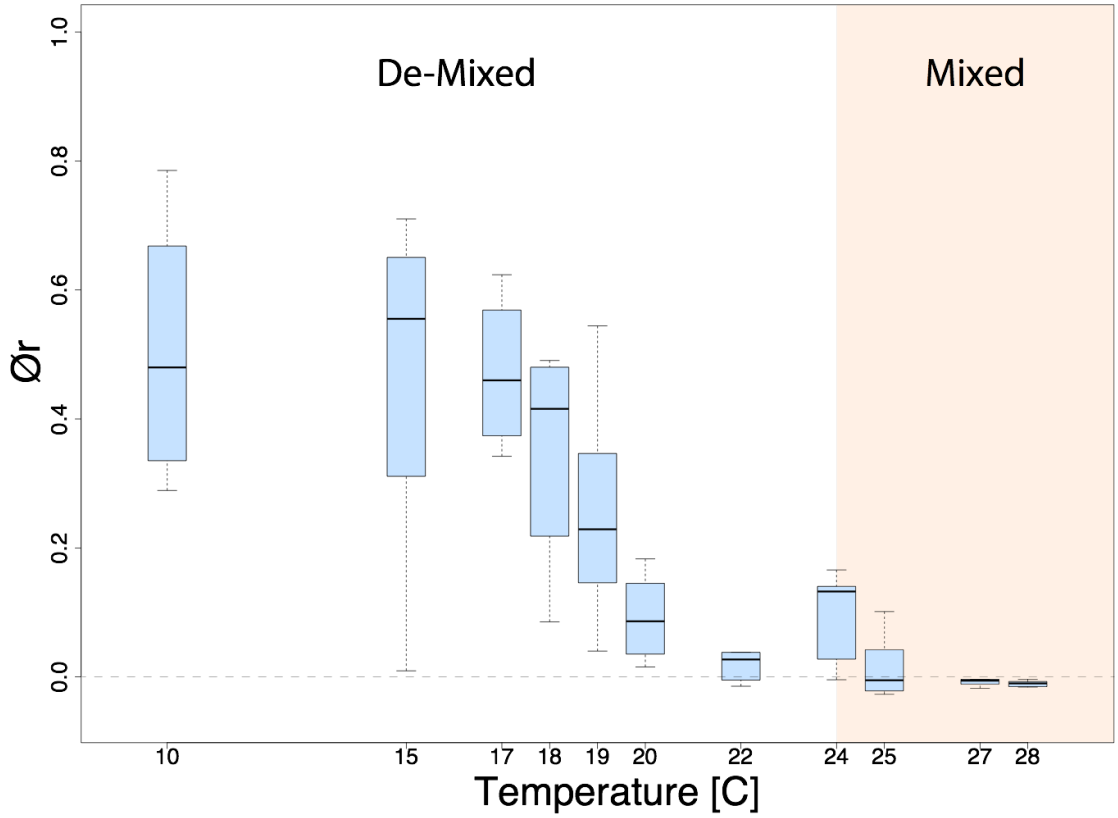


Figure 6. P granules mix following a Smoothed out Phase Separation. Ør is the Order Parameter computed by subtracting droplet concentrations Øin = Dp/Vt and Cytoplasmic Øout = (Cp-Dp)/Ve where ØT=(Dp+Cp) is the total concentration of PGL-1 measured by fluorescence. A smooth transition between De-mixed and Mixed regimes is shown, ranging from 17ºC up to 28ºC where droplets reach a mixed state.

The rationale is that (Ør) would define the boundary at which P granules dramatically change their equilibrium with the cytoplasm from a liquid droplet phase state, into a cytoplasmic mixed state. This means that when the amount of PGL-1::GFP material is more in the cytoplasm or Ør=0 the total amount of PGL-1::GFP is localized predominantly at the interior of P granules.

When comparing the (Ør) values between temperatures it is found that at temperatures below 18°C (Ør) localizes in the droplet phase also described by the number and volume of P granules. When quantifying (Ør) a steep drop off in PGL-

1::GFP is observed from 18ºC to 22ºC following a long and shallow step towards full dissolution up to 28ºC (Figure 6).

For binary mixtures or two component liquid phase separations for example such as PEG/Dextran mixture, there is a steep drop off between the transition density of mixed components and phase separated droplets. However in the C. elegans system we observe that there is a two major transition densities as indicated in Figure 6. This behavior is typically observed in discontinuous phase separations or liquid phase separations with more components that differentially contribute to the droplet phase de-mixing. This mean that as temperature increase, a predominant combination of components mix with the cytoplasm in.ex PGL-3 at 18ºC, leaving other components with slightly different energies of de-mixing in the droplet phase until at 28ºC all droplets mix with the cytoplasm describing a smooth transition for the droplet phase from a de-mixed state towards a mixed state over several temperatures, *in vivo* (Ør) smoothly transitioned between de-mixed droplets to a mixed state over a 9ºC range from 18ºC to 28ºC where all droplets were dissolved (Figure 6).

These results have shown that P granules form via a entropically driven liquid phase separation. However, the type of phase separation does not resemble a sharp transition from liquid droplets to a mixed state typical of binary mixtures and instead, P granules seem to have a smoothed out liquid phase transition. Suggesting that other factors extend P granule dissolution, possibly influenced by an active chemical regulator, that holds up the phase separation process at high temperatures.

An important candidate that could define wether P granules are actively formed by enzymatic activity is the cytosolic phosphatase PPTR-1. This protein has been shown to robust P granule formation during the first asymmetric cell division in *C. elegans* at high temperatures, showing that upon its lack of activity, PGL-1 granules do not form during the polarity onset and firs asymmetric cell division in the worm (Gallo *et al.* 2010a). Following this result, it is important to explore in

more detail the role of temperature on functional mutants of PPTR-1 in respect to the capacity of P granules to liquid phase separate upon a temperature quench.

5.5. P GRANULES DO NOT NEED THE INFLUENCE OF PPTR-1 TO FORM DROPLETS

P granules form as a result of supersaturation of their components PGL-1/3 via a concentration dependent liquid phase separation (Brangwynne *et al.* 2009b). However, it is not clear whether P granules can form solely via weak electrostatic interactions driven by entropy resulting in the formation of liquid P granule droplets. Previous research have shown that P granules require the activity of PPTR-1 to form and remain dynamic during different stages of the first asymmetric cell division (Gallo *et al.* 2010b;Wang *et al.* 2014). Conversely it is possible that a purely physical interaction mediated by P granule proteins could explain the role of temperature in the dissolution of P granules.

To clarify if P granules require the chemical influence of PPTR-1 to form, P0 cell stage embryos were dissected and placed under the microscope at 17ºC and ~25ºC (+/-1ºC) and P granules were observed.

In the fluorescently labelled wild type PGL-1 strain, P granules normally form and segregate towards the posterior end of the embryo at temperatures up to 25ºC (Figure 2). However, in pptr-1(tm3103) mutants, P granules were mixed with the cytoplasm at the normal rearing temperature of 24ºC from POn to Anaphase (Figure 7). To challenge the concept that P granules do not require any chemical action to form, mutant embryos pptr-1(tm3103) were submitted to a temperature quench down to 17ºC and test if P granules would still form.

As expected *pptr-1* (tm3103) mutants formed P granules in POn and they remained across the cell cycle in further stages in respect to Anaphase (Figure

7), suggesting that P granule formation is rescued by the effects of temperature in the cytoplasm of the worm (Figure 7).

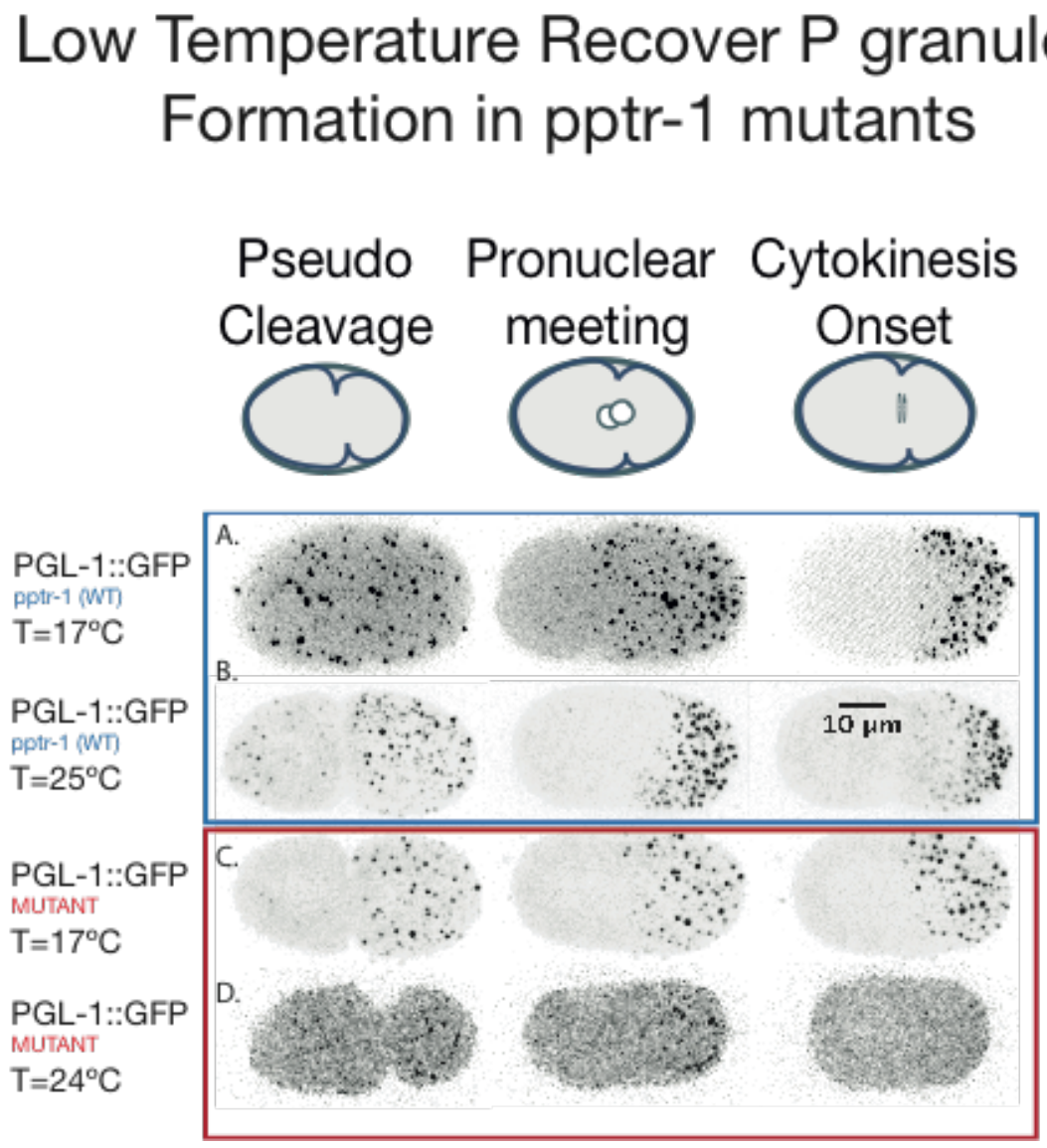


Figure 7. Low Temperature recover P granule formation in pptr-1(3103) P granule null mutants before and after anaphase. P granules dissolve in pptr-1(tm3103) mutant embryos at RT 24°C before and after anaphase. At 17°C, temperature recover P granule formation in the pptr-1 (tm3103) mutant, re-condensing P granules towards the posterior side of the embryo, similarly as wild type embryos do at the limiting temperature of 25°C. Images are maximum projections of embryos at the target temperature, dark spots only represent the detection of a P granule, not its intensity.

The recovery of P granules upon a temperature downshift in *pptr-1* (tm3103) support the idea that temperature is besides concentration the control parameter that drives the formation of P granules via a liquid - liquid phase separation. At the same time, *pptr-1* can not be ruled out on its role as a chemical regulator for the robustness of P granule liquidity at high temperatures.

As temperature can rescue P granule formation in pptr-1 mutants it is possible that temperature not only drive the process of mixing of P granules in the cytoplasm, but also could drive the process of de-mixing as a reversible liquid - liquid phase separation.

5.6. P GRANULES REVERSIBLY MIX AND DE-MIX *IN VIVO*

Previous experiments have shown that the action of *pptr-1* is not essential for P granule formation and that temperature rescues the droplet phase formation. However upon a temperature quench a phase separation could resemble a reversible process and return the P granules from a mixed state to their natural de-mixed state as droplets. The reversibility of the phenomena would tell us that the P granule system is able to pass from a droplet phase to a mixed phase solely by temperature keeping concentration constant, but in addition will reveal additional cues about the physical mechanism of P granule partitioning towards the posterior side of the embryo.

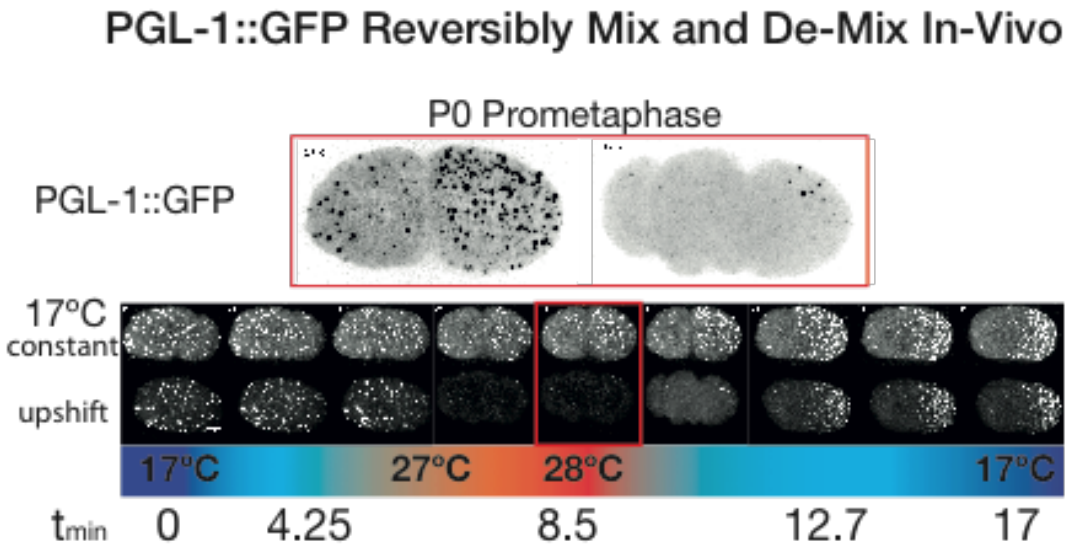


Figure 8. PGL-1 granules reversibly mix and de-mix from the cytoplasm yet polarizing their liquid phase separation. At constant temperatures 17ºC P granules in POn (Polarity Onset) tmin= (0 - 8.5) dissolve everywhere in the cytoplasm tmin=8.5 when temperatures reach 28ºC. Marked in red, a time point of a *C elegans* embryo right at the dissolution point. Upon a temperature quench, P granules de-mix only at the posterior side of the embryo, showing that cytoplasmic partitioning is independent from P granule Phase Separation.

To investigate the reversibility of the P granule system *in vivo*, embryos were imaged in P0 throughout the first asymmetric cell division of the *C. elegans* embryos using the strain TH206 that carries a *gfp* labelled version of *pgl-1::gfp*, and the same strain used for previous experiments described in Figure 2.

A temperature oscillation was applied to the embryo from 17°C to 28°C and back to 17°C plus minus in a total time of 17 minutes of cell division. Results were recorded in a time lapse with a time interval length of 30 sec and 1.2 µW laser power to avoid undesired bleaching effects due the intensive imaging. Stacks were processed as maximum intensity projections of the embryos during P0 cell division up to pro-metaphase as shown in Figure 8 and the presence of P granules and their localization was evaluated upon a temperature upshift from 17°C to 28°C followed by a temperature downshift back 17°C.

P granules can reversibly mix and de-mix in the living cytoplasm of the nematode upon a temperature oscillation during the first asymmetric cell division of the *C. elegans* embryo (Figure 8).

In addition and most surprisingly results suggests, that besides P granules reversibility the recovery of the de-mixed phase occur without loss of the polarity cues that drive P granule phase partitioning towards the poserior. showing that droplets are still segregated towards the posterior side of the embryo dominated by a a controlled dissolution mechanism previously described by Brangwyne *et al*. This indicate that the effects of temperature on the embryo drive P granule droplet de-mixing independently of the polarization cues involved on their anterior - posterior localization throughout cell division such as the cues of MEX-5/6 at the anterior (Tenlen *et al.* 2008).

However, independently of MEX-5/6 evidence here suggest that P granules are indeed formed as a liquid phase separation. To provide supporting evidence of this, P granules in other organs such as oocytes and gonads should also resemble a similar reversible behavior where the influence of embryo polarization factors do not influence the mixing/de-mixing properties of P granules. For this, experiments were performed to assess P granule reversibility in the gonads of the worm, and to evaluate if a difference in P granule cell localization did have an effect on the properties of thermally driven liquid P granules observed in P0 cells.

Gonads from nematode worms with labelled PGL-1::GFP were imaged inside of the worm and followed after a large temperature quench, from 18°C to 32°C. This was done in order to find the boundary, at which gonad P granules respond to temperature for this tissue. Upon a thermal quench, P granules in the gonads revealed that PGL-1::GFP droplets mix with the oocytic cytoplasm at 30°C, gonads were sustained at this temperature for 10 minutes and then downshifted. Surprisingly, when temperature was set back to 18°C, P granules re-condensed in the cytoplasm of the oocytes (Figure 9), demonstrating that a thermally driven phase separation is an phenomena that occur independently of the cell type.

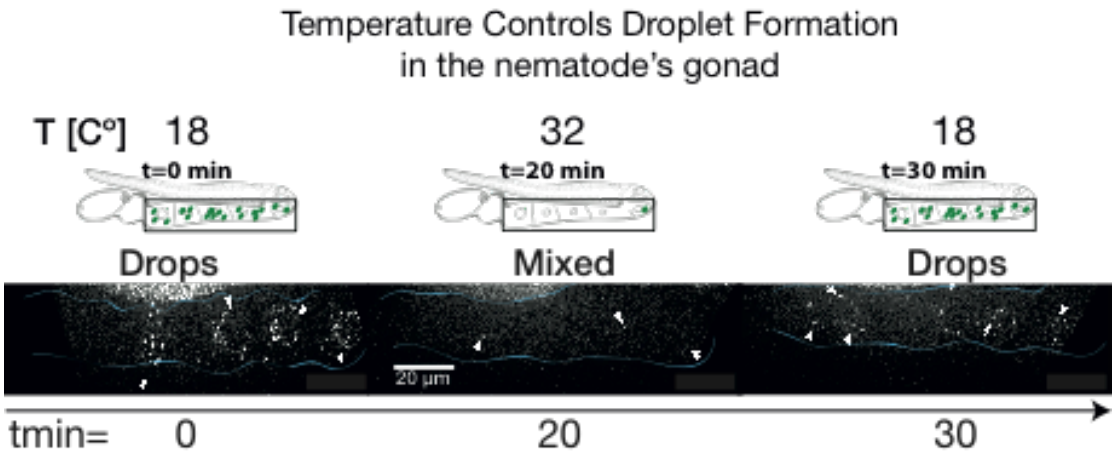


Figure 9. Temperature is a global regulator of P granules independently of the cell type. P granules resemble the same reversible response in the gonad of the nematode, with a higher temperature of mixing (32ºC) after 20 min of exposure, re-condensing at 18ºC.

Embryos in further cell stages were also tested for this thermal response in order to see if the reversible nature of P granule formation was consistent across different cell lineages. To better assess this, an embryo in P2 cell stage was taken and imaged under a wide-field microscopy system with a heating laser adapted to it, in order to dissolve single P granules with an approximated local temperature in the focal point of the laser, of 35ºC. These experiments were done in collaboration with Kreysing lab at the MPI-CBG and the assistance of Mathaeus Mitasch, who built and set the microscope for this experiment.

The dissolution of a single large P granule, that account for approximately 25% of the total phase separated volume was done with spatial accuracy, focusing and pulsing a far-red laser of 1000 nm at 50 Hz for 6 minutes by heating up a single P granule located at the leftmost side of the nucleus of a P2 cell stage embryo. The embryo was placed on the temperature controlled chamber with a background temperature of 18ºC, letting the P granules located at the vicinity of the laser focus in a relatively “cold” state at 18ºC (Figure 10A).

Upon the laser thermal pulse at the leftmost side of the nucleus, P granules dissolved at a local approximated temperature of T 35ºC. Remarkably, an increase

in fluorescence was observed in the neighboring granules, accompanied by an increase in droplet size (Figure 10B).

When the laser was off, the dissolved P granule at the leftmost side of the P2 cell began to re-condense on the surface of the nucleus. At the same time a gain in intensity was observed locally and a reduction in intensity was seen in the other granules, suggesting that P granule material is homogeneously re-distributed among all formed P granule droplets. This observation suggest also, that P granules grow by exchanging material with the cytoplasm via a mechanism known as Ostwald ripening, where droplet material grow at expenses of the soluble material, in this case dissolved PGL-1::GFP.

These experiments indicate that P granule formation is driven by temperature in other cell types, standing as a thermodynamic principle of the liquid state for P granule biology and remain consistent with the supersaturation condition of P granule components, which is necessary to trigger a cytoplasmic liquid-phase separation in *C. elegans*.

As previously shown, in the gonad and in the P2 cell during embryogenesis, P granules are strongly driven to form by temperature. However, since they remain in a cytosolic environment it is possible that this recovery is actively mediated by the enzymatic action of a regulator of the phase separation. Further, that this process is mediated or directly influenced by ATP consumption.

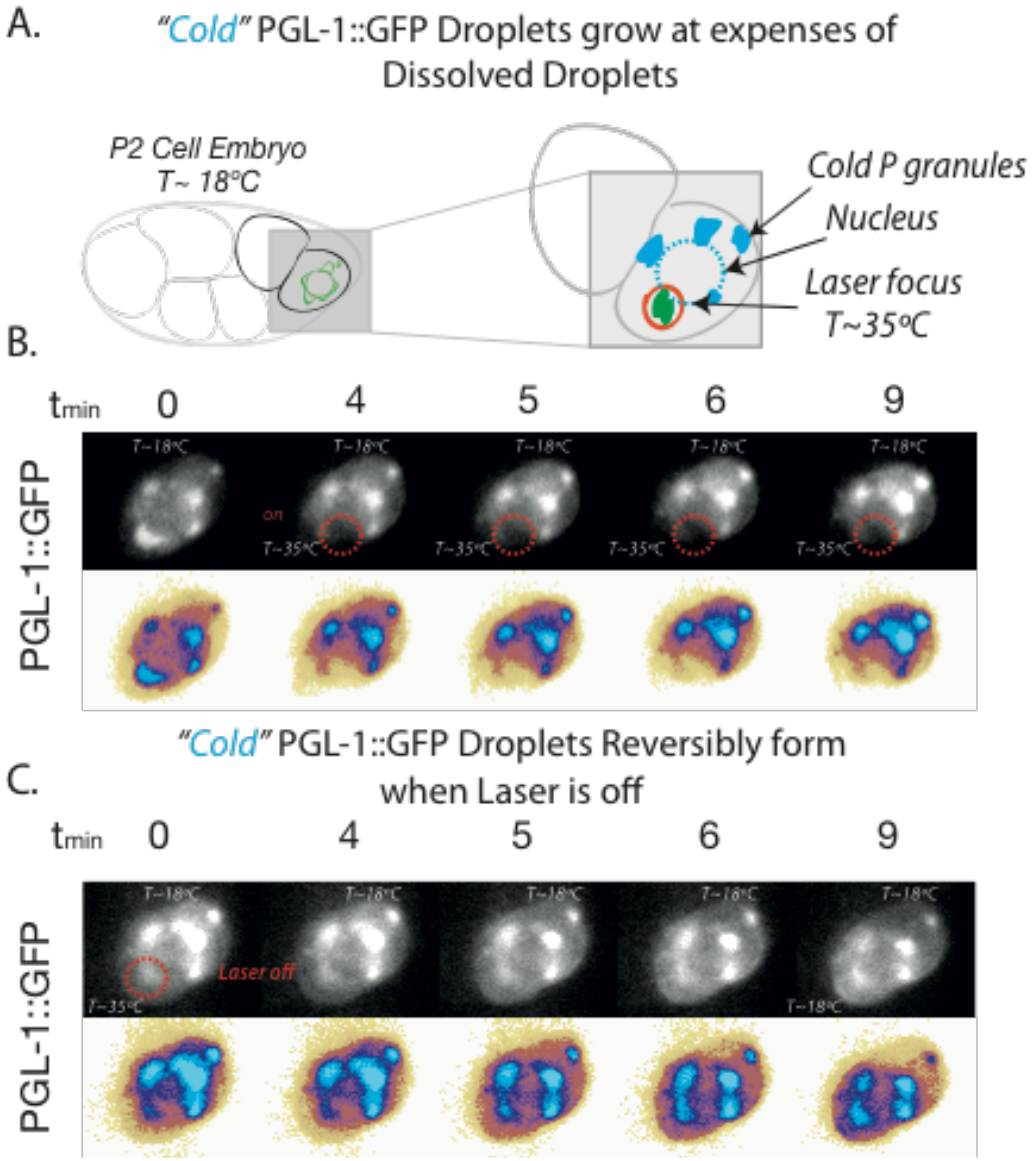


Figure 10. A single P granule in P3 cell stage dissolve upon localized heating with a laser. A. P granules located at the surface of P3 cell nucleus are thermally probed to dissolve with temperature at (35ºC) using a far red laser. B. P granule fully mix with temperature (red dotted circle), leaving the surface of the nucleus devoid of PGL-1::GFP material; the dissolution of the heated up PGL-1::GFP granule increases the intensity of neighboring droplets upper-left where "cold 18ºC" PGL-1::GFP granules remain. C. When laser is off, PGL-1::GFP concentration is re-distributed all over the cytoplasm and P granules de-mix again at the surface of the nucleus.

These experiments show that the formation of a membrane less compartment in the cytoplasm occur via a thermodynamically driven process regulated by

temperature. These findings draw direct evidence that P granule phase separation occurs as a liquid - phase separation.

In order to show that P granules can form on their own, it is necessary to perform an *in vitro* study of PGL-3::GFP, a constitutive P granule protein and evaluate its capacity to form droplets at different temperatures at physiological conditions.

5.7. PGL-3 GRANULES PHASE SEPARATE *IN VITRO* AT PHYSIOLOGICAL CONDITIONS

PGL-1 and PGL-3, together with DEAD box domain helicases GLH-1/4 and other components, are important for P granule formation and constitute the main components of P granules as droplets (Hanazawa *et al.* 2011a).

PGL-3 was chosen to work with because of its efficient purification. PGL-3 was purified and kindly provided for this experiments by Saha et al. (unpublished data from our lab) in vials of 50µM PGL-3::GFP in 300 mM KCl and stored at -80°C.

The formation of PGL-3 droplets *in vitro* and their storage as a stock is problematic in that droplets tend to fuse and form large viscous blobs that covered the walls of the Eppendorf tubes. The use of these vials resulted in inhomogeneous amounts of PGL-3 every time an experiment was performed, due to the variations in the concentration, in part because of the stickiness of the protein to the tubes and in part due to a variable droplet size when collecting the sample.

To solve this problem, it was necessary to devise a starting condition in which the protein remains highly soluble in the buffer then diluting out the salt to trigger the formation of droplets in order to test different temperature conditions under the microscope. As described in the methods section, KCl was used for this purpose at various concentrations to evaluate what is the best concentration to

keep the protein soluble in stock and what is the best condition to start the imaging of the droplets.

The effect of KCl on PGL-3 is chaotropic in nature, meaning that it solubilizes the protein in the buffer at high concentrations. PGL-3 was prepared as described in Methods Figure M4 at 18ºC, in buffer containing 300 mM, 250 mM and 150mM KCl.

PGL-3 readily formed droplets in low salt and pure water. However to set the P granule system into a standard state that could be usable for *in vitro* experiments and trigger droplet formation, it is necessary to use salt as a trigger given that KCl dissolve protein in solution it also allowed to manipulate the droplet formation. For this, a KCl vs protein concentration curve was performed in order to find a concentration of KCL where PGL-3::GFP was mixed. From this point, the formation of droplets could be triggered by adjusting to a working salt concentration suitable for applying different temperature ramps.

Experiments showed that a concentration of 50µM PGL-3::GFP in buffer with 250 mM KCl formed few and small droplets over long incubation times; higher concentrations of KCl showed that 300 mM was a stable and suitable concentration to store the protein at -20ºC. However, sometimes droplets were seen floating in solution in 1 every 10 assays. Higher salt concentrations above 300 mM KCl have unpredictable effects on the liquidity of droplets when salt concentration is lowered. Additional effects of high salt in the droplet phase were also observed, but are outside the scope of this project. Consequently, a concentration of 300 mM KCl was used to store PGL-3::GFP in a mixed state and a final standard concentration of 150 mM KCl was used to stably form droplets together with 50µM of PGL-3::GFP protein.

In order to trigger the reaction, a working buffer was made to dilute the amount of KCl to 150 mM KCl. This amount of salt has been reported to be the physiological concentration of KCl in the nematode cytoplasm (Lamitina *et al.*

2004). Following this, a dilution of 1:1 gave a 25μM PGL-3::GFP with 150 mM KCl and was observed under the microscope at 18°C.

Droplets abundantly condensed in the buffer solution (Figure 11. A,B), indicating that liquid P granules have the ability to form on their own *in vitro* at 18°C. However P granule formation via salting-out of the solution shows that PGL-3::GFP can form droplets at 18°C alone with this solvent. This phenomena is also consistent with observations made by Saha (unpublished results) in the same lab, suggesting that P granules can form in absence of any chemical influence that could drive their nucleation and droplet growth.

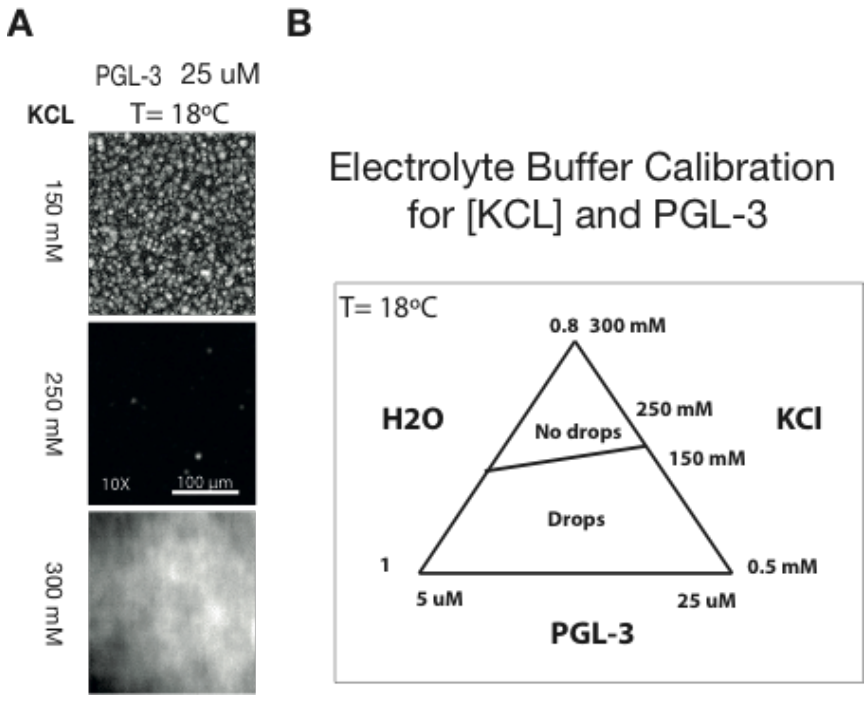


Figure 11. PGL-3 granules form droplets *in vitro*. The formation of liquid compartments is shown in panel A. At concentrations of 150 mM KCl, whereas at higher concentrations, droplet nucleation is poor or null as it occurs when at 300 mM of KCl. Droplets are triggered at concentrations below 150 mM KCl as depicted in the electrolyte buffer diagram for KCl and PGL-3 in water B.

5.8. P GRANULE PHASE SEPARATION IS REVERSIBLE *IN VIVO* AND *IN VITRO*

Following results mentioned above, a comparison between the formation of P granules *in vivo* and *in vitro* was necessary to assess the thermodynamic range at which P granules mix and de-mix. More importantly, their capacity to reversibly form in absence of additional chemical regulators mediated solely as a temperature driven liquid phase separation.

In vitro P granules were triggered to form as described in methods and shown in Figure M4 at a volume fraction of Ø=0.2 of PGL-3::GFP. Droplets were formed at 18ºC and progressively dissolved within 5 minutes when temperature rose up to 32ºC . Following this, temperature was rapidly set back down to T<18ºC, where the formation of droplets was observed after few minutes. Temperature was further lowered to 10ºC in order to speed up the formation of *in vitro* PGL-3 droplets after a mixed state (Figure 7).

P granules could be re-condensed after several temperature quenches following the methods listed above, with PGL-3::GFP mixing and de-mixing with the bulk solvent. This shows that PGL-3 has the property to reversibly mix and de-mix into droplets solely driven by temperature in absence of any chemical regulator. This can be interpreted as PGL-3 molecular interactions being sufficient to drive droplet nucleation and growth of granules *in vitro.*

The temperature sensitivity of P granules and their free and reversible formation of PGL-3 droplets *in vitro* suggest that P granules form in a temperature - concentration dependent manner. Following this, and give the previous evidence that P granules are relevant for the fertility of the worms, it is important to understand if the liquid phase separation of P granules is relevant for the thermal fertile range of the worm. For this, it is important to compare *in vitro* the thermal limits of P granules *in vivo*.

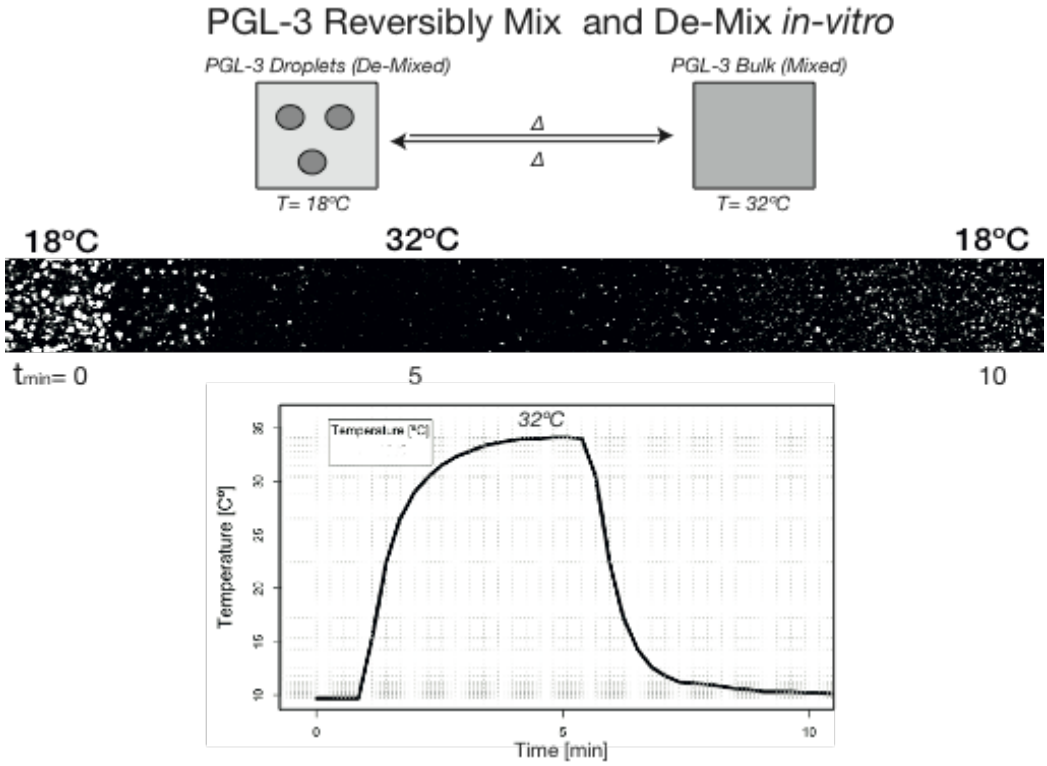


Figure 12. PGL-3 granules reversibly mix and de-mix through the binodal boundary of a liquid phase separation driven by temperature. At 18ºC, PGL-3 droplets form; during a temperature quench P granules pass from a de-mixed state (droplet) to a mixed state (no droplets) at 32ºC, de-mixing again when temperature is shifted down to 18ºC.

5.9. AN *IN VITRO* PHASE DIAGRAM TO COMPARE THE THERMAL LIMITS OF P GRANULES *IN VIVO*

Although the thermodynamic behavior of PGL-3 *in vitro* has shown similarities with PGL-1 granules *in vivo*, it is still unknown if the thermal ranges between both coincide and are meaningful for the maintenance and upkeep of nematode fertility at higher thermal ranges.

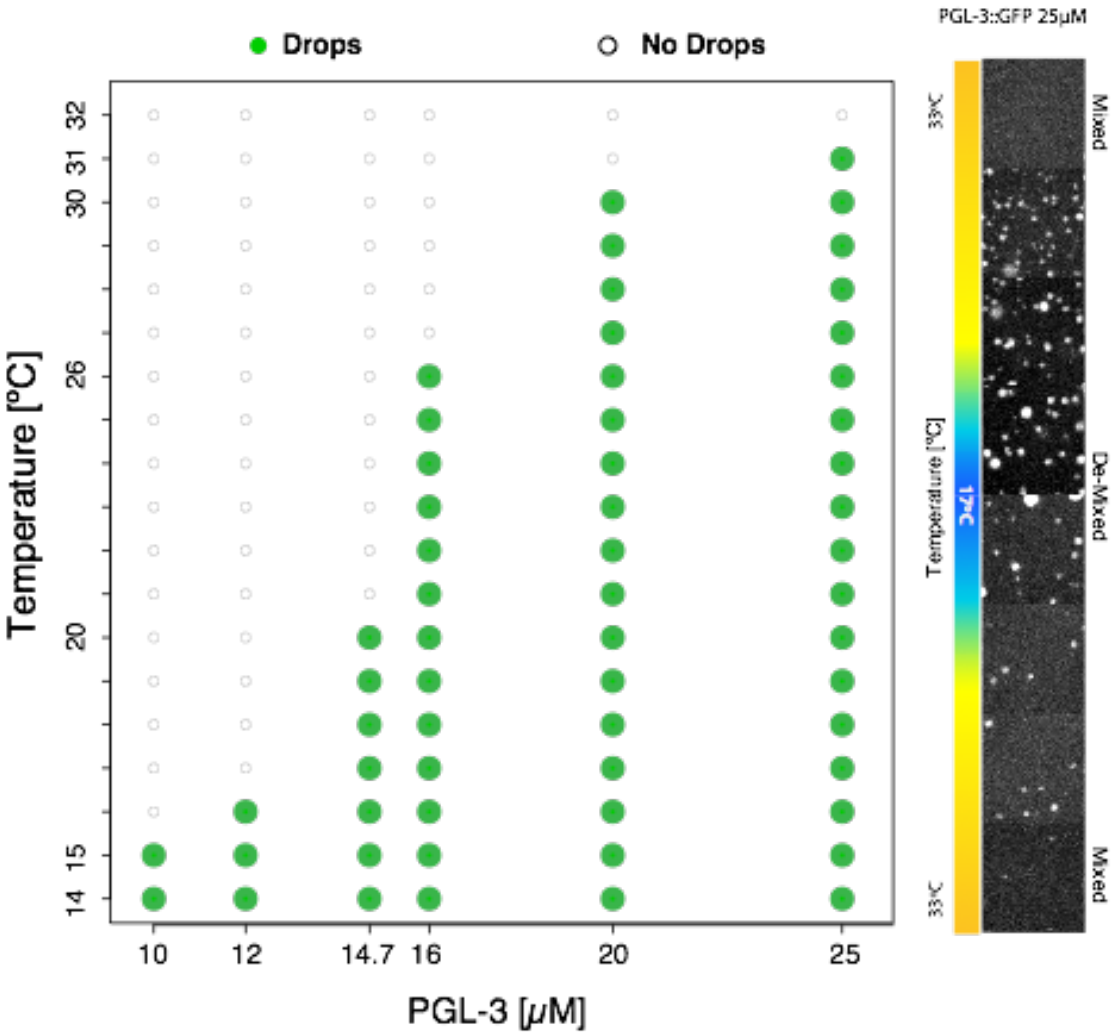


Figure 13. Binary Phase Diagram for PGL-3. PGL-3 droplets form upon a temperature quench the binodal boundary at different concentrations. The relationship between both determines the strength that each molecule has to phase separate from the bulk. As depicted here when PGL-3 concentration reaches 14.6μM it correlates with the thermal limits of de-mixing of *in vivo* PGL-1::GFP granules.

To better compare the thermal range between both systems *in vivo* and *in vitro*, a binary phase diagram was built at different temperatures and concentrations of PGL-3::GFP.

Different concentrations were assayed at different temperatures in order to determine if, at a given temperature and concentration, droplets were formed. Furthermore, we were interested if the binodal line of the phase diagram would intersect at some point with the *in vivo* response to temperature of *C. elegans* fertility.

In summary, results show that PGL-3 is able to form droplets in a concentration dependent manner ranging from 25 µM (ØT=0.5) to 5 µM (ØT=0.1). Moreover, temperature was found to regulate the intermolecular interactions that drive a liquid phase separation *in vitro* at 150 mM KCl, allowing the formation of droplets at temperatures below 29ºC as shown in the phase diagram, or hindering the formation of droplets at temperatures above 32 ºC (Figure 13). The temperature vs concentration range dependence found in the phase diagram is also called Binodal Line in polymer physics, in this case, the binodal line is found to correlate with the thermal range at which P granules mix and de-mix in living worms in the gonads of the worm and embryonic cell stages further P2 (Figure 3 and 13).

6. Discussion

6.2. P GRANULES MIX AND DE-MIX IN A REVERSIBLE MANNER

Two of the major components of P granules are the RNA binding proteins PGL-1 and PGL-3. These proteins are involved in the fertility of the nematode at restrictive temperature limits (25°C) and are also important factors for germ line development (Kawasaki *et al.* 2004;Updike *et al.* 2014). Here, a bridge is made between the thermodynamic aspects of P granules and the fertile thermal limits of *C. elegans*.

P granules have consistently shown to liquid phase separate from the cytoplasm in a process of mixing of their components, driven solely by temperature *in vivo* (Figure 1 and 2). This process occurred only with membrane less compartments, known to resemble liquid behavior such as P granules and different than aggregates such as PATR-1 (P-Bodies) (Figure 1). The mechanisms of P granule formation had been discussed and described as a process of protein association via protein binding, more similarly to a chemically driven process (Hanazawa *et al.* 2011b). This study have found that P granules do not essentially require a chemical reaction between their components to form instead as a liquid phase separation where weak molecular interactions driven by temperature trigger the transition of liquid P granules between a fully soluble mixed state towards a droplet de-mixed state.

In vivo this process does not occur at a single temperature as predicted from Flory Huggins model for Binary mixtures (Figure I1) at a single concentration. This had been shown by the order parameter Ør (Equation 2) that balances the amount of soluble mixed P granule material vs the amount of droplet material and show that P granules coexist with the mixed cytoplasmic phase of PGL-1 over a long temperature range from 18°C (Dp) to 28°C (Cp) (Figure 5).

This finding indirectly implies that P granules liquid phase separate as the sum of several components that contribute to form the droplet phase but with slightly different temperature dependencies in the order of kBT. This mean, that the relative amounts of cytoplasmic PGL-1 pass from the droplet to the cytoplasm at high temperatures, and droplets loose P granule material at higher temperatures (Figure 4 and 6) in a proces more similar to a continuous phase separation.

A continuous phase separation was also observed for gonads and embryos at further stages of embryonic development, and account for additional evidence that confirm that P granules are driven to form, by temperature, regardless of their maturation state or cell type but following a generalized mechanism of mixing/ de-mixing in all cell types that have P granules in their cytoplasm (Figure 3).

P granules are liquid and mix upon a temperature upshift, however results have shown that P granules also de-mix when temperature is down shifted to 17°C showing a reversible temperature response. This phenomena as the previously described mixing by a temperature quench, can also occur in gonads and in further developmental stages of the embryo (Figure 8 and 9), demonstrating as an additional proof of principle, that temperature is an essential factor responsible for P granule formation *in vivo*.

This experiments have wide and at the same time profound interpretations on the mechanisms that form and regulate P granules in time, space and cell type in particular about the physical mechanism that P granule molecules use to phase separate from the cytoplasm in contrast with other soluble components that require the chemical influence of other components to form a compartment such as the centrosomes (Wueseke *et al.* 2012). The formation of P granules *in vitro* and the similarity in their thermal response *in vivo* not only confirms that P granule molecules are able to spontaneously form liquid-like compartments, it also shows that the P granule components PGL-1/3 has the capacity to perform a liquid phase separation as a reversible process, controlled by temperature (Figure 8, 11 and 12).

This is a surprising finding because it points to a mere physical mechanism of formation that follows soft matter thermodynamical principles of a liquid phase separation. Ruling out the possibility that P granules need a chemical modification influenced by ATP consumption mediated by other factors such as PPTR-1 or MBK-2 for their formation (Wang *et al.* 2014).

6.3. CONCENTRATION AND THE SPATIAL CONTROL OF P GRANULES

An important aspect for a liquid phase separation to occur is its concentration dependence. Concentration lower the energy of mixing in a solution, driving droplets to come out of solution or precipitate under supersaturated cytoplasmic conditions. PGL-1 and PGL-3 are highly concentrated cytosolic proteins (personal communication, Mathias Mann lab), that have shown a consistent thermal response in regard with the formation of cytoplasmic droplets.

The formation of nucleation points as an initial state for P granule formation is in general caused by inhomogeneities in solution, that move forward the nucleation boundary for droplet formation reaching a critical radius where droplets grow.

These nuclei reach a critical droplet size over long time ranges, that initiate an exponential phase of growth where the phase separation is triggered to occur via the phenomena of Ostwald Ripening (Taylor 1998). The rate of growth and the effect of temperature in terms of the nucleating capacity of P granules remains largely overlooked in this work, however it is impossible to ignore that in spite P granules phase separate like liquids in the cytoplasm, they seem to be spatio-temporally regulated upon a temperature quench observed during P0 cell division, being nucleated only at the posterior end of the embryo and not everywhere in the cytoplasm as expected to occur in a two component phase separated system (Figure 8 and 10). This suggests that there are cytoplasmic regulators, that also control the dissolution of P granules in agreement with the findings by (Brangwynne *et al.* 2009b).

This results indicate, that droplets could form following two possible scenarios:

1) P granules dissolve during Polarity Onset upon a temperature quench and re-condense everywhere in the cytoplasm of the embryo, independently and asynchronously in regard to the cell cycle stage, “forgetting” the cytoplasmic polarity cues nucleating everywhere in the cytoplasm.

2) P granules homogeneously dissolve upon a temperature quench during Polarity Onset that upon a downshift in temperature reversibly form towards the posterior end of the embryo, “memorizing” the final spatial configuration of P granule condensation in synchrony with the cell cycle stage.

The scenario 1 would predict, that an active chemical regulator is only necessary to position P granules towards the posterior and it would break down upon high temperature, letting P granule components form droplets at the posterior solely by the entropy of de-mixing at low temperatures.

On the other hand the scenario 2 predict, that there is a certain cytoplasmic factor that is temperature “insensitive” and “memorize” the positional information, where P granules should nucleate in synchrony with the cell cycle progression. Even after a full dissolution of P granules upon a temperature quench as high as 28°C.

In accordance with other studies, MEX-5/6 proteins are important candidates that could explain the correct positioning of P granules regardless of a thermal quench in the scenario 2. Mainly because MEX-5/6 forms a cytoplasmic gradient opposite to P granule phase separation during POn, that is established by the end of anaphase, that could create a dissolution zone at the anterior where P granules can not form droplets (Figure 14).

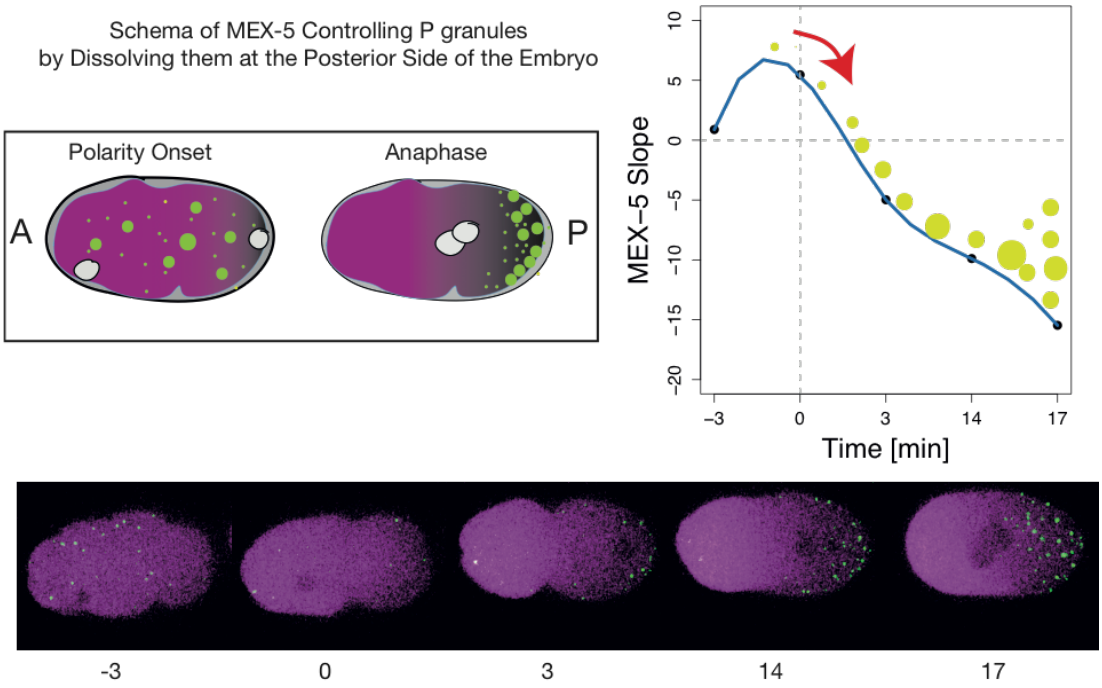


Figure 14. MEX-5 is a cytoplasmic gradient that regulate the formation of P granules at the Posterior. During Polarity Onset, MEX-5 is dispersed throughout the cytoplasm of the P0 cell, in co-existence with P granule droplets; over time (0 - 17 min), MEX-5 accumulates at the posterior, creating a gradient dissolving P granules at the anterior side of the embryo.

This supports the idea that a second regulator induce the dissolution of P granules at the anterior, in order to de-mix droplets at the posterior side of the embryo, triggered by the direct interaction of MEX-5/6 gradient (Schubert *et al.* 2000) with P granules as proposed by Brangwyne et al 2009.

Previous experiments of my own authorship are supporting this evidence, and demonstrate that MEX-5 is indeed temperature insensitive in regard with its spatial regulation at a given temperature upshift of 31°C as shown in Figure 15.

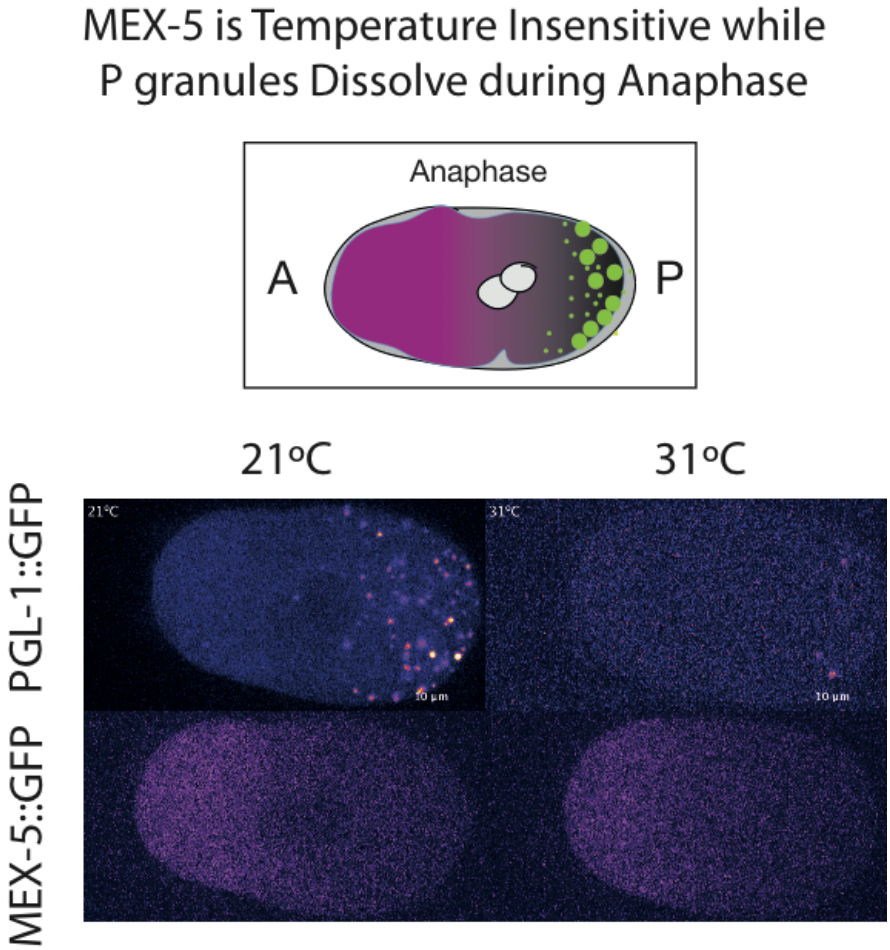


Figure 15. MEX-5 is a P granule regulator, insensitive to temperatures higher than 27ºC. MEX-5 is spatio-temporally anti-correlated with P granule formation, forming a gradient towards the anterior part of the embryo. P granules dissolve independently from MEX-5 gradient, being restricted at the posterior part of the embryo.

On the other hand PGL-1 remains restricted to form droplets towards the posterior end of the embryo, even after a temperature downshift being delimited by the spatial region where P granules can still mix and de-mix (Figure 8). The interaction with MEX-5/6 is still an open question on P granule spatio-temporal regulation in terms of how this affect P granule liquid phase separation.

6.4. THE ROLE OF OTHER CHEMICAL REGULATORS

It has been shown that P granules can reversibly change between a droplet or a mixed state driven by temperature *in vivo,* being spatio-temporally regulated by factors such as MEX-5/6 in the P0 cell embryo (Figure 10 and 15). However the

formation of P granules could also depend or be actively regulated by the chemical activity of the cytoplasm from within the droplet phase, thus extending the chemical resilience of the droplet phase when necessary in order to perform their biological function possibly associated with their phase separated state, as shown for other Ribo-Nucleo Protein assemblies forming RNP granules (Hubstenberger *et al.* 2013;Nott *et al.* 2015).

In regard to this, studies on the biochemistry of P granule formation have shown that their formation is favored by the activity of PP2A phosphatase holoenzyme, mediated by the subunits *pptr-1/2* (Gallo 2010). Mutations in *pptr-1* hinder the nucleation of P granule growth during Anaphase throughout all the embryo, meanwhile un-specifically disrupting the partitioning of P granule formation towards the posterior end of the embryo, while promoting P granule dissolution during the first asymmetric division onwards.

The question raised here is, what is the role of PPTR-1 as a P granule chemical regulator? As shown here, temperature is able to control the formation of P granules and experiments here support the hypothesis that PPTR-1 is not required to nucleate P granules as evidenced in the *pptr-1* (tm3103) mutant at temperatures lower than 24ºC to 17ºC with P granules nucleating and growing towards the posterior side of the embryo (Figure 7). However the action of PPTR-1 may be important in order to extend the robustness of the liquid state of P granules rendering them more “robust” at high temperature regimes in synergy with other P granule components besides PGL-1/3, as shown for MEG-3/4 (Leacock and Reinke 2008;Wang *et al.* 2014), which may act as transducers of chemical signals that can modulate the droplet phase.

Altogether, these results confirm that P granule droplets are a mixture of multiple components showing a different thermal response than a discontinuous phase separation and instead showing a smoothed out dissolution response (Figure 6). One possibility for this observation is that each component of the droplet phase contribute to the energy of de-mixing to form P granule droplets, thus extending the binodal line of the liquid phase separation for several degrees above the

mixing point, as is the case for a multi component liquid mixture (Rosenholm 2014).

The regulation of P granule droplets is relevant, because depending on the number of components their liquidity may change to other states, from liquid to gel-like or to solid. This behavior is still an open question for the biology of P granules, specially during their maturation throughout development, because the control of the liquid phase could determine the activity at the interior of the droplet phase in respect with the cytoplasmic milieu.

The maturation of a multi-component P granule system may vary the physical properties of P granules, such as viscosity and surface tension, henceforth change the time-scales at which other biochemical processes occur at the interior vs the exterior of the droplet phase at a given developmental time during germ line formation.

The role of other chemical regulators have important consequences, not only to understand how to control P granules spatio-temporally in composition and localization, but also to understand how this process of maturation is relevant for the survival of *C. elegans* at higher ecological fertile thermal limits.

6.5. ECOLOGICAL RELEVANCE OF P GRANULE PHASE SEPARATION

Nematodes can survive from 10°C to 30°C for long exposures. However their fertility is severely compromised if temperature reach limits above 27°C where their soma can develop and grow, but nematodes become sterile. P granule mixing and de-mixng range correlates with the worm's fertile range from 15°C to 18°C. P granules remain liquid de-mixed droplets with more than Ør ≥ 50% of PGL-1 content in the droplet phase (Figure 6), this matches with the worm's most fertile between at 18°C to 19°C (~400 brood size). On the other hand when temperatures rose above 20ªC, P granules start to mix with the cytoplasm and worms, steadily loose their fertility when temperatures reach 27°C. At this point

PGL-1 concentration inside of the droplet phase is less than Vf ≤ 0.005% of the total PGL-1 volume.

This correlation indicate, that P granule droplets are driven to de-mix and mix with the cytoplasm by the influence of temperature increasing the energy of mixing upon a temperature shock.

P granules mix with the cytoplasm over a long temperature range, resembling a discontinuous liquid phase separation. However here is shown that P granule droplets formation can be chemically regulated by the chemical action of a P granule regulator that extends the lifetime of P granules as liquid cytoplasmic droplets above the binodal line of single P granule components such as PGL-3. The chemical transduction of PPTR-1, together with the influence of spatial other regulators for instance MEX-5/6 and MEG-3/4 could spatio-temporally control the droplet phase formation while extending the thermal range where P granules can form up to 27°C, suggesting that P granules play a critical role on the temperature adaptation of nematodes at higher thermal fertile limits.

7. Concluding Remarks

In *Caenorhabditis elegans* P granule phase separation is controlled by temperature. P granules are able to phase separate within a broad thermal range, highlighting a continuous character of P granules, as a liquid - liquid phase separation. This discontinuous phase separation occur in the range between 18°C to 26°C where P granules have shown to mix as confirmed evaluating the order parameter (Ør) of the PGL-1::GFP fractions in cytosolic phase and the droplet phase. When temperature reaches 27°C P granules remarkably mix with the cytoplasm at the same temperature at which the cell cycle rate looses their linear relationship with temperature reported as the break up in the Arrhenius temperature dependency of the cell cycle rates.

Evaluation of PGL-1 and PGL-3 *in vivo* and *in vitro* accordingly, have showed remarkable similarities in the thermal response that they resemble in particular, the reversible nature and ability to form P granules upon a temperature quench. This reversible behavior occur *in vivo* and as shown here it draws conclusive evidence that indicate that temperature can drive P granules to reversibly mix and de-mix in the cytoplasm of gonads and embryos.

In vitro experiments showed similar results in minimal buffer without ATP or any enzyme that could affect droplet formation. With a temperature response that match P granules *in vivo* in both, temperature range and concentration.

These results together with *in vitro* experiments, showed that P granule-like compartments can be de-mixed or formed solely by the effects of temperature. Indicating that temperature sets the boundaries at which entropy drive PGL-3 to form a P granule-like compartment *in vitro*. And that this mechanism operates in a similar manner *in vivo.* Here conclusive evidence showed that the dominant mechanism in P granule condensation is a liquid-phase separation driven by temperature and concentration.

The absence of any biochemical regulator in the *in vitro* assays is an important hallmark that confirm that weal intermolecular interactions between PGL-3 are necessary to create a P granule like compartment in vitro and in vivo. This observation led to the conclusion that P granules do not require any chemical factors that influence their formation or confer active reversible condensation *in vivo* or *in vitro* as evidenced in the recovery of P granules in mutants for PPTR-1 phosphatases at 17ºC.

It is important to highlight that even though P granules can form on their own, P granules regulators could function as “hot and cold” regulators, that upkeep P granule formation at different thermal ranges. These regulators such as PPTR-1, MBK-1 or MEG-1/4 could chemically modify the droplet phase hence extend the range at which P granules phase separate. This can occur simultaneously with the spatio-temporal control of P granules droplet formation via a controlled dissolution/condensation mechanism driven solely by concentration throughout the first asymmetric cell division at different thermal limits.

This hypothesis is consistent as well with other hypothesis in relation with the influence of MEX-5/6 on P granules spatio-temporal control together with the downstream control of other cytoplasmic gradients on the embryo partitioning of the cytoplasm (Wu and Griffin 2014;Wu *et al.* 2015b). The interplay between these regulators is not only important in the control of the phase separation per se (Brangwynne *et al.* 2009b), but also in the role of extending for instance, the viscosity and surface tension of P granules (Elbaum-Garfinkle *et al.* 2015) over and/or under certain thermal boundaries where the nematode has evolved and by doing so increasing its fitness.

This study opens a new perspective to study biological adaptations in the germ line of extreme organisms and their evolutionary trends related with the tuning of physical parameters in order to set their survival at extreme conditions.

Perhaps the most interesting question to be answered yet is, what are the thermodynamical requirements for temperature adaptation?

When considering the cytoplasm as a liquid, a matrix gel, or a viscoelastic material it results evident that pressure and temperature play an important role in the tuning of its properties, specially the interaction and activities of proteins as it occurs in complex polymeric mixtures, with the difference that in the cytoplasm these polymers are peptides that exert control and direct the formation of several cytoplasmic structures such as spindles, microtubules, mitochondria and other organelles.

What keeps all these structures thermodynamically together with special regard with organisms that live in extreme conditions?

Bibliography

Adhikari, B. N., D. H. Wall and B. J. Adams (2009). "Desiccation survival in an Antarctic nematode: molecular analysis using expressed sequenced tags." Bmc Genomics **10**(1).

Begasse, M. L., M. Leaver, F. Vazquez, S. W. Grill and A. A. Hyman (2015a). "Temperature Dependence of Cell Division Timing Accounts for a Shift in the Thermal Limits of C. elegans and C. briggsae." CellReports: 1-8.

Begasse, M. L., M. Leaver, F. Vazquez, S. W. Grill and A. A. Hyman (2015b). "Temperature Dependence of Cell Division Timing Accounts for a Shift in the Thermal Limits of C. elegans and C. briggsae." CellReports: 1-8.

Beshore, E. L., T. J. McEwen, M. C. Jud, J. K. Marshall, J. A. Schisa and K. L. Bennett (2011). "C. elegans Dicer interacts with the P-granule component GLH-1 and both regulate germline RNPs." Developmental biology **350**(2): 370-381.

Biosca, J. A., F. Travers and T. E. Barman (1983). "A jump in an Arrhenius plot can be the consequence of a phase transition." FEBS letters **153**(1): 217-220.

BOLTE, S. and F. P. CORDELIÈRES (2006). A guided tour into subcellular colocalization analysis in light microscopy. Journal of Microscopy, Blackwell Publishing Ltd. **224:** 213-232.

Boswell, R. (1985). tudor, a gene required for assembly of the germ plasm in Drosophila melanogaster. Cell. **43:** 97-104.

Boveri, T. (1887). Zellen-Studien., G. Fischer,.

Brangwynne, C. P., C. R. Eckmann, D. S. Courson, A. Rybarska, C. Hoege, J. Gharakhani, F. Julicher and A. A. Hyman (2009a). Germline P Granules Are Liquid

Droplets That Localize by Controlled Dissolution/Condensation. Science. **324:** 1729-1732.

Brangwynne, C. P., C. R. Eckmann, D. S. Courson, A. Rybarska, C. Hoege, J. Gharakhani, F. Julicher and A. A. Hyman (2009b). "Germline P Granules Are Liquid Droplets That Localize by Controlled Dissolution/Condensation." Science **324**(5935): 1729-1732.

Campbell, A. C. and D. L. Updike (2015). CSR-1 and P granules suppress sperm-specific transcription in the C. elegans germline. Development, The Company of Biologists Limited. **142:** 1745-1755.

Carré, C. and D. Carré (2015). "Origin of Germ Cells, Sex Determination, and Sex Inversion in Medusae of the Genus Clytia (Hydrozoa, Leptomedusae): The Influence of Temperature." Journal of Experimental Zoology: 1-10.

Cary, S. C., T. Shank and J. Stein (1998). Worms bask in extreme temperatures. Nature, Nature Publishing Group. **391:** 545-546.

Chowdhury, S., B. W. Jarecki and B. Chanda (2015). "Molecular Determinants of Temperature Dependent Gating of Ion Channels." Biophysical Journal **108**(2): 426a.

Decker, M., S. Jaensch, A. Pozniakovsky, A. Zinke, K. F. O'Connell, W. Zachariae, E. Myers and A. A. Hyman (2011). Limiting amounts of centrosome material set centrosome size in C. elegans embryos. Curr. Biol. **21:** 1259-1267.

Elbaum-Garfinkle, S., Y. Kim, K. Szczepaniak, C. C.-H. Chen, C. R. Eckmann, S. Myong and C. P. Brangwynne (2015). "The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics." PNAS **112**(23): 7189-7194.

Gallo (2010). "Cytoplasmic partitioning of P granule components is not required to specify the germline in C. elegans." Science **330**(6011): 1685-1689.

Gallo, C. M., E. Munro, D. Rasoloson, C. Merritt and G. Seydoux (2008). Processing bodies and germ granules are distinct RNA granules that interact in C. elegans embryos. Dev. Biol. **323:** 76-87.

Gallo, C. M., J. T. Wang, F. Motegi and G. Seydoux (2010a). Cytoplasmic partitioning of P granule components is not required to specify the germline in C. elegans. Science.

Gallo, C. M., J. T. Wang, F. Motegi and G. Seydoux (2010b). "Cytoplasmic partitioning of P granule components is not required to specify the germline in C. elegans." Science.

Goehring, N. W., C. Hoege, S. W. Grill and A. A. Hyman (2011). "PAR proteins diffuse freely across the anterior-posterior boundary in polarized C. elegans embryos." The Journal of Cell Biology **193**(3): 583-594.

Goehring, N. W. and A. A. Hyman (2012). "Organelle Growth Control through Limiting Pools of Cytoplasmic Components." Current biology : CB **22**(9): R330-339.

Grill, S. W. and A. A. Hyman (2005). Spindle Positioning by Cortical Pulling Forces. Developmental cell. **8:** 461-465.

Haataja, M. and C. Brangwynne (2014). Nucleolar assembly and growth are governed by a concentration-dependent phase transition., ... OF THE CELL.

Hanazawa, M., M. Yonetani and A. Sugimoto (2011a). "PGL proteins self associate and bind RNPs to mediate germ granule assembly in C. elegans." The Journal of Cell Biology.

Hanazawa, M., M. Yonetani and A. Sugimoto (2011b). PGL proteins self associate and bind RNPs to mediate germ granule assembly in C. elegans. J Cell Biol.

Hirsh, D. (1979). Temperature Sensitive Maternal Effect Mutants of Early Development in Caenorhabditis elegans, Elsevier**:** 149-165.

Hoege, C., A.-T. Constantinescu, A. Schwager, N. W. Goehring, P. Kumar and A. A. Hyman (2010). "LGL can partition the cortex of one-cell Caenorhabditis elegans embryos into two domains." Current biology : CB **20**(14): 1296-1303.

Howard, J. (2006). Elastic and damping forces generated by confined arrays of dynamic microtubules. Phys Biol, IOP Publishing. **3:** 54-66.

Hubstenberger, A., S. L. Noble, C. Cameron and T. C. Evans (2013). "Translation Repressors, an RNA Helicase, and Developmental Cues Control RNP Phase Transitions during Early Development." Developmental cell **27**(2): 161-173.

Hyman, A. A. and C. P. Brangwynne (2011). "Beyond stereospecificity: liquids and mesoscale organization of cytoplasm." Developmental cell **21**(1): 14-16.

Hyman, A. A. and J. G. White (1987). Determination of cell division axes in the early embryogenesis of Caenorhabditis elegans. J Cell Biol, Rockefeller Univ Press. **105:** 2123-2135.

JOHNSTON, I., H. GUDERLEY, C. FRANKLIN, T. CROCKFORD and C. KAMUNDE (1994). "Are Mitochondria Subject to Evolutionary Temperature Adaptation." Journal of Experimental Biology **195**: 293-306.

Kawasaki, I., A. Amiri, Y. Fan, N. Meyer, S. Dunkelbarger, T. Motohashi, T. Karashima, O. Bossinger and S. Strome (2004). "The PGL family proteins associate with germ granules and function redundantly in Caenorhabditis elegans germline development." Genetics **167**(2): 645-661.

Kawasaki, I. I., Y. H. Y. Shim, J. J. Kirchner, J. J. Kaminker, W. B. W. Wood and S. S. Strome (1998a). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans. Cell. **94:** 635-645.

Kawasaki, I. I., Y. H. Y. Shim, J. J. Kirchner, J. J. Kaminker, W. B. W. Wood and S. S. Strome (1998b). "PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans." Cell **94**(5): 635-645.

Kim-Ha, J., J. L. Smith and P. M. Macdonald (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell. **66:** 23-35.

Kühn, T., T. O. Ihalainen, J. Hyväluoma, N. Dross, S. F. Willman, J. Langowski, M. Vihinen-Ranta and J. Timonen (2011). Protein Diffusion in Mammalian Cell Cytoplasm. PloS one, Public Library of Science. **6:** e22962.

Kumamoto, J., J. K. Raison and J. M. Lyons (1971). Temperature “breaks” in Arrhenius plots: A thermodynamic consequence of a phase change. J Theor Biol. **31:** 47-51.

Lamitina, S. T., R. Morrison, G. W. Moeckel and K. Strange (2004). Adaptation of the nematode Caenorhabditis elegans to extreme osmotic stress. American Journal of Physiology - Cell Physiology, American Physiological Society. **286:** C785-791.

Leacock, S. W. and V. Reinke (2008). "MEG-1 and MEG-2 Are Embryo-Specific P-Granule Components Required for Germline Development in Caenorhabditis elegans." Genetics **178**(1): 295-306.

Lithgow, G. J., T. M. White, D. A. Hinerfeld and T. E. Johnson (1994). "Thermotolerance of a long-lived mutant of Caenorhabditis elegans." Journal of Gerontology **49**(6): B270-B276.

Mahowald, A. P. (1962). Fine structure of pole cells and polar granules in Drosophila melanogaster. Journal of Experimental Zoology, Wiley Subscription Services, Inc., A Wiley Company. **151:** 201-215.

Nott, T. J., E. Petsalaki, P. Farber, D. Jervis, E. Fussner, A. Plochowietz, T. D. Craggs, D. P. Bazett-Jones, T. Pawson, J. D. Forman-Kay and A. J. Baldwin (2015). "Phase Transition of a Disordered Nuage Protein Generates Environmentally Responsive Membraneless Organelles." Molecular cell **57**(5): 936-947.

Orsborn, A. M., W. Li, T. J. McEwen, T. Mizuno, E. Kuzmin, K. Matsumoto and K. L. Bennett (2007). "GLH-1, the C. elegans P granule protein, is controlled by the JNK KGB-1 and by the COP9 subunit CSN-5." Development **134**(18): 3383-3392.

Patel, A., H. O. Lee, L. Jawerth, S. Maharana, M. Jahnel, M. Y. Hein, S. Stoynov, J. Mahamid, S. Saha, T. M. Franzmann, A. Pozniakovski, I. Poser, N. Maghelli, L. A. Royer, M. Weigert, E. W. Myers, S. Grill, D. Drechsel, A. A. Hyman and S. Alberti (2015). A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. Cell, Elsevier. **162:** 1066-1077.

Peukes, J. and T. Betz (2014). "Direct measurement of the cortical tension during the growth of membrane blebs." Biophysical Journal **107**(8): 1810-1820.

Pietzsch, T., S. Preibisch, P. Tomancak and S. Saalfeld (2012). ImgLib2-generic image processing in Java. Bioinformatics, Oxford University Press. **28:** 3009-3011.

Prodon, F. F., J. J. Chenevert, C. C. Hébras, R. R. Dumollard, E. E. Faure, J. J. Gonzalez-Garcia, H. H. Nishida, C. C. Sardet and A. A. McDougall (2010). Dual mechanism controls asymmetric spindle position in ascidian germ cell precursors. Development. **137:** 2011-2021.

Reber, S. and A. A. Hyman (2011). Samurai Sword Sets Spindle Size. Cell. **147:** 1224-1225.

Reber, S. B., J. Baumgart, P. O. Widlund, A. Pozniakovsky, J. Howard, A. A. Hyman and F. Juelicher (2013). XMAP215 activity sets spindle length by controlling the total mass of spindle microtubules. Nat. Cell Biol., Nature Publishing Group. **15:** 1116-+.

Rosenholm, J. B. (2014). Phase equilibriums, self-assembly and interactions in two-, three- and four medium-chain length component systems. Adv Colloid Interface Sci. **205:** 9-47.

Sackmann, E. (2014). "Endoplasmatic reticulum shaping by generic mechanisms and protein-induced spontaneous curvature." Advances in colloid and interface science **208**: 153-160.

Schabhüttl, S., P. Hingsamer, G. Weigelhofer, T. Hein, A. Weigert and M. Striebel (2013). Temperature and species richness effects in phytoplankton communities. Oecologia. **171:** 527-536.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods, Nature Publishing Group. **9:** 676-682.

Schindelin, J., C. T. Rueden, M. C. Hiner and K. W. Eliceiri (2015). The ImageJ ecosystem: An open platform for biomedical image analysis. Mol. Reprod. Dev. **82:** 518-529.

Schneider, C. A., W. S. Rasband and K. W. Eliceiri (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods, Nature Publishing Group. **9:** 671-675.

Schubert, C. M., R. Lin, C. J. de Vries, R. H. Plasterk and J. R. Priess (2000). MEX-5 and MEX-6 function to establish soma/germline asymmetry in early C. elegans embryos. Molecular Cell. **5:** 671-682.

Sengupta, M. S., W. Y. Low, J. R. Patterson, H.-M. Kim, A. Traven, T. H. Beilharz, M. P. Colaiácovo, J. A. Schisa and P. R. Boag (2013). ifet-1 is a broad-scale translational repressor required for normal P granule formation in C. elegans. J. Cell. Sci., The Company of Biologists Ltd. **126:** 850-859.

Shirae-Kurabayashi, M., T. Nishikata, K. Takamura, K. J. Tanaka, C. Nakamoto and A. Nakamura (2006). Dynamic redistribution of vasa homolog and exclusion of somatic cell determinants during germ cell specification in Ciona intestinalis. Development, Company of Biologists. **133:** 2683-2693.

Shtilerman, M. D., a. Tomas T Ding and J. Peter T Lansbury (2002). Molecular Crowding Accelerates Fibrillization of α-Synuclein: Could an Increase in the Cytoplasmic Protein Concentration Induce Parkinson's Disease?†. Biochemistry, American Chemical Society. **41:** 3855-3860.

Stewart, M. P., J. Helenius, Y. Toyoda, S. P. Ramanathan, D. J. Muller and A. A. Hyman (2011). "Hydrostatic pressure and the actomyosin cortex drive mitotic cell rounding." Nature **469**(7329): 226-230.

Strome, S. and D. Updike (2015). "Specifying and protecting germ cell fate." Nature reviews. Molecular cell biology **16**(7): 406-416.

Strome, S. and W. B. Wood (1982). Immunofluorescence Visualization of Germ-Line-Specific Cytoplasmic Granules in Embryos, Larvae, and Adults of Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America. **79:** 1558-1562.

Taylor, N., S. Elbaum, H. Stone and C. Brangwynne (2015). Assembly, Properties and Function of Synthetic Phase-Separated RNA/Protein Organelles. APS March Meeting 2015**:** 1218P.

Taylor, P. (1998). Ostwald ripening in emulsions. Adv Colloid Interface Sci. **75:** 107-163.

TEAM, R. C. (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Tenlen, J. R., J. N. Molk, N. London, B. D. Page and J. R. Priess (2008). "MEX-5 asymmetry in one-cell C. elegans embryos requires PAR-4- and PAR-1-dependent phosphorylation." Development **135**(22): 3665-3675.

Thomson, T., N. Liu, A. Arkov, R. Lehmann and P. Lasko (2008). "Isolation of new polar granule components in Drosophila reveals P body and ER associated proteins." Mechanisms of development **125**(9-10): 865-873.

Updike, D. L., S. J. Hachey, J. Kreher and S. Strome (2011). "P granules extend the nuclear pore complex environment in the C. elegans germ line." The Journal of Cell Biology **192**(6): 939-948.

Updike, D. L., A. K. a. a. Knutson, T. A. Egelhofer, A. C. Campbell and S. Strome (2014). "Germ-Granule Components Prevent Somatic Development in the C. elegans Germline." Current Biology **24**(9): 970-975.

Vanreusel, A., G. Fonseca, R. Danovaro, M. C. Da Silva, A. M. Esteves, T. Ferrero, G. Gad, V. Galtsova, C. Gambi, V. Da Fonsêca Genevois, J. Ingels, B. Ingole, N.

Lampadariou, B. Merckx, D. Miljutin, M. Miljutina, A. Muthumbi, S. Netto, D. Portnova, T. Radziejewska, M. Raes, A. Tchesunov, J. Vanaverbeke, S. Van Gaever, V. Venekey, T. N. Bezerra, H. Flint, J. Copley, E. Pape, D. Zeppilli, P. A. Martinez and J. Galeron (2010). "The contribution of deep-sea macrohabitat heterogeneity to global nematode diversity." Marine Ecology **31**(1): 6-20.

Voronina, E., A. Paix and G. Seydoux (2012). "The P granule component PGL-1 promotes the localization and silencing activity of the PUF protein FBF-2 in germline stem cells." Development **139**(20): 3732-3740.

Vourekas, A., Y. Kirino and Z. Mourelatos (2010). Elective affinities: a Tudor-Aubergine tale of germline partnership. Genes & Development, Cold Spring Harbor Lab. **24:** 1963-1966.

Wang, J. T., J. Smith, B.-C. Chen, H. Schmidt, D. Rasoloson, A. Paix, B. G. Lambrus, D. Calidas, E. Betzig and G. Seydoux (2014). "Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in C. elegans." eLife **3**: e04591.

Woodruff, J. B., O. Wueseke and A. A. Hyman (2014). Pericentriolar material structure and dynamics. Philos. Trans. R. Soc. Lond., B, Biol. Sci., The Royal Society. **369:** -20130459.

Woodruff, J. B., O. Wueseke, V. Viscardi, J. Mahamid, S. D. Ochoa, J. Bunkenborg, P. O. Widlund, A. Pozniakovsky, E. Zanin, S. Bahmanyar, A. Zinke, S. H. Hong, M. Decker, W. Baumeister, J. S. Andersen, K. Oegema and A. A. Hyman (2015). Regulated assembly of a supramolecular centrosome scaffold in vitro. Science, American Association for the Advancement of Science. **348:** 808-812.

Wu, Y. and E. Griffin (2014). The MEX-5/6 concentration gradient controls PIE-1 segregation in the cytoplasm of the C. elegans zygote. Molecular Biology of the Cell. **25**.

Wu, Y., H. Zhang and E. E. Griffin (2015a). Coupling between cytoplasmic concentration gradients through local control of protein mobility in the C. elegans

zygote. Molecular Biology of the Cell, American Society for Cell Biology**:** mbc.E15-05-0302.

Wu, Y., H. Zhang and E. E. Griffin (2015b). "Coupling between cytoplasmic concentration gradients through local control of protein mobility in the C. elegans zygote." Molecular Biology of the Cell: mbc.E15-05-0302.

Wueseke, O., J. Bunkenborg, J. S. Andersen and T. Hyman (2012). Centrosome maturation starts at centrioles: Implications from a clear cytoplasmic separation of key players of centrosome maturation in C. elegans. Molecular Biology of the Cell. **23**.

Yajima, M., E. A. Gustafson, J. L. Song and G. M. Wessel (2014). "Piwi regulates Vasa accumulation during embryogenesis in the sea urchin." Developmental Dynamics **243**(3): 451-458.

Zhu, L., S. Weber, J. Berry, N. Vaidya, M. Haataja and C. Brangwynne (2015). Phase Transitions in the Nucleus: the functional implications of concentration-dependent assembly of a Liquid-like RNA/Protein Body. APS March Meeting 2015**:** 1078P.

Zwicker, D., A. A. Hyman and F. Julicher (2015). "Suppression of Ostwald ripening in active emulsions." Phys Rev E Stat Nonlin Soft Matter Phys **92**(1-1): 012317.